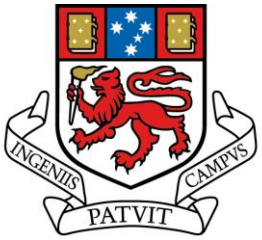

UNIVERSITY
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Cell Expansion Coordinates Leaf Vein and Stomatal Density

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BSc (Hons)

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Declaration

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Statement of co-authorship

The following people and institutions contributed to the publication of work undertaken as part of this thesis:

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Chapter 2 is published:

Carins Murphy MR, Jordan GJ, Brodribb TJ (2014) Acclimation to humidity modifies the link between leaf size and the density of veins and stomata. *Plant, Cell and Environment* **37**, 124-131.

Candidate was the primary author (75 %), author 2 assisted with designing the research and writing the manuscript (18.75 %) and author 3 assisted with data analysis and writing the manuscript (6.25 %).

Chapter 3 has been submitted to *Annals of Botany*:

Carins Murphy MR, Jordan GJ, Brodribb TJ (in review) The coordination of veins and stomata in both herbs and woody species is dominated by cell expansion not cell differentiation.

Candidate was the primary author (75 %), author 2 assisted with designing the research and writing the manuscript (18.75 %) and author 3 assisted with data analysis and writing the manuscript (6.25 %).

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Abstract

The efficiency with which water is delivered through leaf tissue to sites of evaporation (the leaf hydraulic conductance) is an important determinant of photosynthetic capacity. This is because inadequate water supply forces stomata to close due to plant desiccation. The resulting reduction in stomatal conductance restricts carbon dioxide (CO_2) uptake. An oversupply of water, on the other hand, results in unprofitable investment in vascular tissue. Accordingly, water supply is strongly correlated with the demand for water generated by stomata. Recent work suggests that this balance is achieved in a tropical angiosperm tree by a simple developmental mechanism in which changes to leaf size co-regulate vein and stomatal density (major determinants of water supply and demand, respectively). However, the generality of this mechanism is unknown. Thus, in this thesis I set out to establish whether there is a general developmental mechanism that allows plants to maintain a constant ratio between leaf vein and stomatal density. In doing so the following specific research questions were investigated:

1. Does acclimation to high and low VPD (vapour pressure difference) modify the relationships between vein density, stomatal density and leaf size? (Chapter 2)

2. Does leaf or epidermal cell expansion coordinate vein and stomatal density in herbs and woody angiosperms? (Chapter 3)

3. Does epidermal cell expansion coordinate vein and stomatal density in older plant lineages like ferns? (Chapter 4)

4. Is vein density directly regulated by epidermal cell expansion? (Chapter 5)

The difference in vapour pressure between leaf tissue and the atmosphere determines how much water is transpired at any given stomatal conductance. Thus, I hypothesised in Chapter 2 that plants grown under high VPD would exhibit a

modified relationship between vein and stomatal density resulting in maintenance of homeostasis in leaf water potential such that there is either a decrease in stomatal density and no change in vein density, or no change in stomatal density and an increase in vein density. This would also disrupt previously observed relationships with leaf size. However, contrary to my hypothesis, I found a small but coordinated increase in vein and stomatal density in plants grown under high VPD compared with those grown under low VPD. Furthermore, densities of veins and stomata were independent of large VPD-induced changes to leaf size and were instead limited by epidermal cell size (which was fairly insensitive to VPD). This suggests that significant modification of epidermal cell size is required to produce large changes in vein and stomatal density.

Thus, to further investigate whether leaf expansion or epidermal cell expansion coordinates vein and stomatal density more generally among plants I examined relationships between vein density, stomatal density, leaf size and epidermal cell size across a diverse range of woody and herbaceous angiosperms grown under sun and shade conditions (Chapter 3). Contrary to the original premise that differential leaf expansion coordinates vein and stomatal density, I found that leaf size was independent of epidermal cell size in most cases and that relationships between vein density, stomatal density and epidermal cell size were well described by modelled relationships that assume veins and stomata are passively 'diluted' by epidermal cell expansion. These results demonstrate that coordination of vein and stomatal density in angiosperms is driven by their co-variation with epidermal cell size.

While this 'passive dilution' mechanism seems to be common among angiosperms, it is not known when it appeared in the vascular plant phylogeny. Furthermore, older plant lineages like ferns may employ different mechanisms to coordinate vein and stomatal density because they differ from angiosperms in both current water relations physiology and evolutionary history. Contrary to this expectation, I found that relationships between vein density, stomatal density and epidermal cell size across

four ferns species were very similar to those previously observed across angiosperm species (Chapter 4). However, there was little plasticity in these traits within fern species and changes to stomatal density across species were actively regulated by stomatal index, as well as epidermal cell size. Despite this, epidermal cell size was a strong determinant of vein and stomatal density in ferns (explaining 55.5 % of the variation in stomatal density versus the 44.5 % explained by stomatal index). Thus, ferns (like angiosperms) appear to use the co-variance of vein and stomatal density with epidermal cell expansion to maintain a constant ratio between the abundance of veins and stomata in the leaf. This suggests that the 'passive dilution' mechanism may be an ancient feature of vascular plants that co-regulates these tissues.

In Chapters 3 and 4 it was proposed that coordination of vein and stomatal density is achieved through a 'passive dilution' mechanism in which densities of veins and stomata are co-regulated by epidermal cell size. However, unlike stomata, leaf veins are spatially isolated from the epidermis and it is not known whether they are directly regulated by epidermal cell expansion. To investigate this I tested whether relationships between vein density and epidermal cell size in a wild type genotype of *Arabidopsis* (Col-0) and seven other genotypes with modified forms of genes that affect both stomatal development and epidermal cell size were the same as modelled relationships that assume veins are passively 'diluted' by epidermal cell expansion (Chapter 5). Vein density in wild type plants was correlated with abaxial epidermal cell size in a way that was consistent with the 'passive dilution' mechanism (despite some deviation from modelled relationships). However, vein density was independent of variation in epidermal cell size among mutant and transgenic genotypes. This suggests that epidermal cell size in these genotypes was modified independently from the rest of the leaf in spite of prior evidence that cell sizes are correlated within leaves. Thus, vein density is not causally linked to epidermal cell expansion. Instead the relationship between vein density and epidermal cell size in wild type plants reflects developmental factors that affect both mesophyll and epidermal cells, suggesting that adaptation favours coordination of veins and stomata over independent development of these tissues.

Thus, the overarching finding of this thesis is that the ‘dilution’ of veins and stomata by differential epidermal cell expansion (and perhaps mesophyll cell expansion) appears to be a general mechanism capable of maintaining a constant ratio between vein and stomatal density both within and across a diverse range of vascular plant species. Leaf cells expand more in the shade than the sun, and more in some species compared with others, which increases the space between veins and stomata concomitantly reducing their density. These relationships provide an insight into how plants construct leaves that can efficiently replace transpired water and maintain maximum carbon assimilation for the minimum investment in vein and stomatal infrastructure. Achieving this maximises the energetic return for investments made during leaf construction increasing the energy available for growth and reproduction.

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Chapter 1
General introduction

All multicellular organisms invest energy in constructing the tissues necessary for ongoing growth and reproduction. Thus, ensuring that energetic return exceeds investment is critical for survival, and maximising the size of the excess could have direct impacts on fitness by increasing the resources available for reproduction. Achieving this is particularly important for plants because they produce all the chemical energy they need by harvesting the radiant energy of sunlight and using it to convert water and carbon dioxide (CO_2) into carbohydrates. Accordingly, trade-offs between leaf traits related to structural or biochemical investment and photosynthetic gain have been observed across plant species from around the world suggesting that plants utilise a continuum of leaf investment strategies that ensure maximum energetic return for investment (Wright *et al.* 2004; Reich 2014). Leaf hydraulic traits are thought to contribute to the position of species along this continuum because of their influence over leaf carbon and water fluxes (Reich 2014). Because carbon assimilation rates are sensitive to leaf water status and the xylem tissue that irrigates the leaf is costly to produce, a potentially important trade-off exists between investment in leaf veins and carbon uptake. Constructing leaves that uphold a constant ratio between leaf vein density (total vein length per unit area) and stomatal density (total stomata per unit area) ensures that water supply and transpirational demand are balanced and that investment in vein infrastructure does not exceed the minimum required for maximum photosynthetic output (Brodribb and Jordan 2011; Carins Murphy *et al.* 2012). However, the mechanisms that facilitate the close developmental control of these separate tissues are not well understood. Thus, this thesis aims to establish whether plants share a general mechanism for maintaining a balance between leaf vein and stomatal density.

1.1 Coordination of leaf vein and stomatal density as a mechanism for balancing water supply with transpirational demand

Plants take up CO_2 through microscopic pores in the leaf surface called stomata. Water vapour is lost (transpired) at the same time through the stomata due to the difference in vapour pressure between leaf tissue and the relatively drier atmosphere (the VPD). If transpired water is not replenished by water delivered through plant veins from the soil, stomata close to prevent leaf desiccation. This in turn restricts the uptake of CO_2. Thus, an undersupply of water results in diminished photosynthetic returns due to stomatal closure. On the other hand, an over investment in leaf veins is also suboptimal because there are energetic costs associated with vein synthesis (Lambers and Poorter 1992). Therefore, understanding how plants efficiently construct leaves capable of supplying enough water to allow stomata to open to the optimal aperture set by leaf photosynthetic biochemistry (Medlyn *et al.* 2011), and thus, maintain maximum rates of carbon uptake suitable for the leaf photosynthetic capacity is an important goal of plant development research. Because stomata regulate the demand for water (the stomatal conductance) and the leaf vasculature determines the efficiency of water supply to evaporative surfaces near the stomata (the leaf hydraulic conductance) (Sack and Frole 2006; Brodribb *et al.* 2007), one might predict that coordination of vein and stomatal density balances water supply with transpirational demand. This may occur either within individual plants or species such that plastic changes to vein and stomatal density are coordinated in response to environmental conditions and/or across species as a result of selection to uphold a constant ratio between these traits. Why one would expect vein and stomatal density to be balanced in this way will now be expanded upon in more detail.

As mentioned previously, water loss from leaves is primarily due to evaporation from within the leaf and in most cases occurs almost entirely through the stomata (Kerstiens 1996). The rate of transpiration (E) is therefore determined by stomatal conductance (g_s) and the VPD (van den Honert 1948):

$$E = g_s \times VPD. \quad (1.1)$$

Uncontrolled water loss can lower leaf water potential to the extent that air bubbles form in the veins blocking water flow to the leaves (Zimmermann 1983). Thus, to regulate transpiration, plants adjust stomatal conductance either instantaneously by opening and closing stomata (Raschke 1975) or during leaf development by adjusting stomatal size and/or stomatal density (Woodward 1986; Aasamaa *et al.* 2001; Hetherington and Woodward 2003). This thesis will be primarily concerned with developmental changes to stomatal size and density.

To meet transpirational demand, plants take up water from the soil and deliver it through the xylem to sites of evaporation near the stomata. Xylem is specialised tissue made up of rigid-walled cells that forms a continuous network of veins connecting the roots to the leaves. Van den Honert (1948) proposed that water movement through plant veins is analogous to the movement of electrons through an electric circuit. The resulting model for the flow of water through plant tissues has received almost universal acceptance amongst plant physiologists. In this model, the physical characteristics of the hydraulic pathway between soil and the sites of evaporation determine the resistance to water flow. Thus, as water travels through the plant body its water potential declines as a function of the resistance (or conductance) of plant tissue and the rate of transpiration:

$$\Delta\Psi_P = E/K_P, \quad (1.2)$$

where $\Delta\Psi_P$ is the difference in water potential between the soil and the leaf ($\Psi_S - \Psi_L$), E is the transpirational flux and K_P is the plant hydraulic conductance. In well-hydrated soils, this relationship can be simplified further as the sensitivity of stomatal conductance to water potential is relatively consistent across plant groups (Brodribb

and Holbrook 2003) meaning $\Delta\Psi_P$ is rather conservative. This implies that hydraulic conductance dictates maximum rates of transpiration in most plants. Furthermore, because the leaf represents a substantial bottleneck for water flow through the plant body – accounting for at least 30 % of whole plant hydraulic resistance ($R_P = 1/K_P$) – leaf hydraulic conductance is representative of whole plant hydraulic conductance (Tyree *et al.* 1993; Yang and Tyree 1994; Nardini and Salleo 2000; Sack *et al.* 2003). This is verified by the strong correlation between leaf hydraulic conductance and maximum rates of CO_2 assimilation across a broad range of terrestrial plants (Brodribb *et al.* 2005; Brodribb *et al.* 2007). In addition, the correlation between leaf vein density and leaf hydraulic conductance (Sack and Frole 2006; Brodribb *et al.* 2007) suggests that vein density regulates plant hydraulic performance and thus maximum rates of CO_2 assimilation.

Therefore, the limitations of a shared pathway for water and CO_2 through the stomatal pore, along with the energetic costs of producing and maintaining leaf veins (Lambers and Poorter 1992) and stomata, raises the expectation that coordination of vein and stomatal density balances water supply with transpirational demand. Coordination of vein and stomatal density has been observed in response to light intensity within individuals (sun and shade leaves) and within species (sun and shade plants) in woody angiosperms from tropical and temperate habitats (Brodribb and Jordan 2011; Carins Murphy *et al.* 2012; Martins *et al.* 2014). This allows plants to approach a balance whereby investment in the leaf vein network is enough that stomata are supplied with the minimum amount of water required to allow them to open to the optimal aperture set by leaf photosynthetic biochemistry under well watered conditions (Medlyn *et al.* 2011). The stomata can therefore provide the CO_2 required for maximum photosynthesis, but with the minimum amount of vein and stomatal infrastructure. Under these circumstances plants are thought to optimise photosynthetic gain for the energy invested in the construction and ongoing maintenance of the vascular and stomatal systems (Brodribb and Jordan 2011; Franks *et al.* 2012).

1.2 Differential leaf expansion can enable hydraulic acclimation to sun and shade

Shade leaves of woody angiosperms are often larger and have lower densities of veins and stomata than sun leaves from the same species or individual (Bergen 1904; Wylie 1949; Wylie 1951; Abrams and Kubiske 1990). This inverse relationship suggests that veins and stomata may be passively 'diluted' or 'concentrated' by light-induced changes to leaf size, such that vein and stomatal density are inversely proportional to √leaf size and leaf size, respectively (Zwieniecki *et al.* 2004). Thus, the co-variance of vein and stomatal density with leaf size could facilitate their coordinated acclimation to variable light conditions. In support of this hypothesis, it was found that light-induced changes to leaf size (mediated by epidermal cell size) co-regulate vein and stomatal density in the subtropical angiosperm tree *Toona ciliata* M.Roem. (Carins Murphy *et al.* 2012). Thus, in this woody species differential leaf expansion under high and low light can facilitate the coordinated development of veins and stomata, and as a result, balance water supply with transpirational demand. However, the generality of this mechanism is not known.

1.3 Structure of thesis

Thus, in this thesis I set out to establish whether there is a general developmental mechanism that allows plants to maintain a constant ratio between leaf vein and stomatal density. In doing so the following specific research questions were investigated:

1. Does acclimation to high and low VPD modify the relationships between vein density, stomatal density and leaf size? (Chapter 2)

2. Does leaf or epidermal cell expansion coordinate vein and stomatal density in herbs and woody angiosperms? (Chapter 3)

3. Does epidermal cell expansion coordinate vein and stomatal density in older plant lineages like ferns? (Chapter 4)

4. Is vein density directly regulated by epidermal cell expansion? (Chapter 5)

1.4 Overview of chapters

The four experimental chapters of this thesis that address these questions are composed of self-contained units, presented in the style of scientific journal articles. Each contains an introduction to the literature providing an outline of the potential contribution of the investigation to the field, the results and a discussion of the findings and conclusions. Chapter 2 has been published as a peer-reviewed article (Carins Murphy *et al.* 2014) and Chapter 3 has been submitted. A brief introduction to each chapter is provided below.

In Chapter 2 I investigate whether acclimation to high and low VPD modifies relationships between vein density, stomatal density and leaf size. While leaf conductances to liquid- and vapour-phase water are determined by leaf anatomy (vein density and stomatal density/size) (Woodward 1986; Aasamaa *et al.* 2001; Hetherington and Woodward 2003; Brodribb *et al.* 2007), it is the leaf environment that translates these conductances into water fluxes. In particular, atmospheric humidity or VPD governs how much water is transpired at any given stomatal conductance (see equation 1.1). For example, if two leaves with the same density of stomata were exposed to either high or low VPD under well-watered conditions, transpiration would be greatest in the leaf experiencing high VPD. Thus, we would expect a leaf grown under high VPD to require either a change in the relationship between vein and stomatal density (i.e. a higher vein density and no change in stomatal density or a lower stomatal density and no change in vein density) or a reduction in stomatal aperture to maintain homeostasis in leaf water potential. However, the costs associated with having closed stomata mean that the most

efficient use of resources would occur if developmental adjustments mitigated the need for stomatal closure. Hence, the relationship between vein and stomatal density may be modified during leaf acclimation to variable VPD in order to maintain a balance between water supply and transpirational demand in the most energy efficient manner. Modification of this relationship would also disrupt the covariance of vein and stomatal density with leaf size previously observed in *T. ciliata* (Carins Murphy *et al.* 2012). Thus, in Chapter 2, I examine whether these relationships remain proportional in *T. ciliata* plants grown under high and low VPD.

In Chapter 3 I test whether leaf or epidermal cell expansion coordinates vein and stomatal density in herbs and woody angiosperms. To date, the coordination of vein and stomatal density by differential leaf expansion under high and low light (mediated by epidermal cell size) has only been observed in woody angiosperms (Carins Murphy *et al.* 2012). However, herbs may have different capacities and mechanisms for leaf acclimation. There is evidence that the leaves of some herbaceous species undergo increased expansion when grown in the shade (Hughes 1959; Willmot and Moore 1973; Ciha and Brun 1975; Rawson and Craven 1975; Young and Smith 1980; Thomas *et al.* 2003), while in others sun leaves are larger than shade leaves (Gay and Hurd 1975; Dengler 1980; Kalve *et al.* 2014). If increased leaf expansion acts to decrease vein and stomatal density in unison in herbs (as has been observed in woody species) then herbs with larger sun leaves would be maladapted to both sun and shade conditions. Because sun leaves in this case would have lower densities of veins and stomata than shade leaves, either shade leaves would be oversupplied with veins and stomata (and therefore impose energetic costs on the plant) or the sun leaves would be undersupplied and susceptible to the adverse effects of high evaporative demand under high light. This suggests that the passive response of vein and stomatal density to differential leaf expansion observed in woody angiosperms (Carins Murphy *et al.* 2012) may be absent in herbaceous plants. However, in Chapter 2 of this thesis I demonstrate that plasticity in vein and stomatal density is independent of large VPD-induced changes to leaf size and is instead

limited by epidermal cell size. Therefore, another explanation for herbaceous sun leaves sometimes being larger than shade leaves is that vein and stomatal density are regulated by differential epidermal cell expansion rather than leaf expansion. This hypothesis is tested in Chapter 3.

In Chapter 4 I examine whether epidermal cell expansion coordinates vein and stomatal density in older plant lineages like ferns. Consistent with the hypothesis described above, I demonstrate in Chapter 3 that coordination of vein and stomatal density is regulated by differential epidermal cell expansion (not leaf expansion) across a diverse range of woody and herbaceous angiosperms. However, it is unknown how deep in the vascular plant phylogeny this 'passive dilution' mechanism is rooted. Proportional relationships between vein density, stomatal density and epidermal cell size may be absent from early-branching vascular plant clades such as ferns because they differ from angiosperms in both current water relations physiology and evolutionary history. Thus, ferns may have different mechanisms to maximise the benefit-cost ratio between photosynthetic return and energetic investment in vein infrastructure. There is some evidence that ferns can adaptively regulate the ratio between vein and stomatal density (Zhang *et al.* 2014), however, it is not known whether these relationships are maintained by the co-variance of vein and stomatal density with epidermal cell expansion as has been observed in angiosperms. In Chapter 4, therefore, I compare relationships between vein density, stomatal density and epidermal cell size across ferns with those previously observed across angiosperms.

In Chapter 5 I use mutant and transgenic genotypes to test whether vein density is directly regulated by epidermal cell expansion. Chapters 2, 3 and 4 present evidence to suggest that epidermal cell expansion co-regulates vein and stomatal density in a diverse sample of woody angiosperms, herbaceous angiosperms and ferns. However, unlike stomata, leaf veins are spatially isolated from the epidermis and it is not known whether vein density is causally linked to epidermal cell expansion. Recent

advances in the understanding of stomatal development have produced a suite of genotypes that specifically alter stomatal density, stomatal index, and epidermal cell size in the model species *Arabidopsis thaliana* (L.) Heynh. (Dow and Bergmann 2014). Isolated control of cellular development in the epidermis allowed us to observe whether changes in external tissues remained coordinated with developmental programs in adjacent and physiologically linked internal tissues. It was predicted that stomatal density would be regulated by genetic changes to stomatal index and, more generally, to epidermal cell expansion, while vein density would remain closely tied to genotype-specific differences in epidermal cell size. Chapter 5 specifically tests this prediction.

Finally, in Chapter 6 (General discussion) I synthesise my findings and discuss their implications in relation to the relevant literature. I also suggest directions for future research.

Chapter 2

Acclimation to humidity modifies the link between leaf size and the density of veins and stomata

This chapter has been published as:

Carins Murphy MR, Jordan GJ, and Brodribb TJ (2014) Acclimation to humidity modifies the link between leaf size and the density of veins and stomata. *Plant Cell and Environment* **37**, 124-131.

2.1 Abstract

The coordination of veins and stomata during leaf acclimation to sun and shade can be facilitated by differential epidermal cell expansion so large leaves with low vein and stomatal densities grow in shade, effectively balancing liquid- and vapour-phase conductances. As the difference in vapour pressure between leaf and atmosphere (VPD) determines transpiration at any given stomatal density, we predict that plants grown under high VPD will modify the balance between veins and stomata to accommodate greater maximum transpiration. Thus, we examined the developmental responses of these traits to contrasting VPD in a woody angiosperm (*Toona ciliata* M. Roem.) and tested whether the relationship between them was altered. High VPD leaves were one-third the size of low VPD leaves with only marginally greater vein and stomatal density. Transpirational homeostasis was thus maintained by reducing stomatal conductance. VPD acclimation changed leaf size by modifying cell number. Hence, plasticity in vein and stomatal density appears to be generated by plasticity in cell size rather than cell number. Thus, VPD affects cell number and leaf size without changing the relationship between liquid- and vapour-phase conductances. This results in inefficient acclimation to VPD as stomata remain partially closed under high VPD.

2.2 Introduction

One of the major questions in plant development is how plants efficiently construct leaves capable of supplying enough water to replace transpirational loss. Central to this is the balance between leaf vein density, which is a critical factor in hydraulic conductance and therefore water supply (Sack and Frole 2006; Brodribb *et al.* 2007), and stomatal density and size, which dictates maximum stomatal conductance and therefore maximum transpiration (Franks and Beerling 2009). Leaf vein density and stomatal density remain proportional during acclimation to sun and shade, both among populations and within individuals in a number of woody angiosperm species (Brodribb and Jordan 2011; Carins Murphy *et al.* 2012). This relationship reflects an

efficient balance between investment in liquid and vapour conductances in the leaf. Thus, the most efficient utilization of vein and stomatal investment occurs when the supply of water to evaporative surfaces near the stomata is just enough to maintain fully open stomata under saturating light conditions in the field (Franks *et al.* 2012). If the maximum evaporative capacity of the epidermis is greater than the capacity of the vascular system to maintain leaf hydration, then stomata will be unable to remain open (Salleo *et al.* 2000; Brodribb and Holbrook 2003). Hence, exceeding a certain stomatal density will incur costs (in the construction and regulation of stomata) that are not matched by greater photosynthetic yield. Likewise, having excess venation will also be inefficient due to both the high carbon cost of synthesizing lignin (the main component of leaf venation) (Lambers and Poorter 1992) and the loss of photosynthetic potential resulting from the displacement of photosynthetic tissue by vascular tissue.

Coordination between water transport and stomatal systems allows leaves to maintain an efficient balance between water use and carbon acquisition while accommodating the different rates of photosynthesis and evaporation experienced under high and low irradiance (Brodribb and Jordan 2011; Carins Murphy *et al.* 2012). It remains to be seen, however, whether other environmental pressures that influence plant water use induce similar developmental responses. Although plasticity has been observed in both stomatal density and vein density in response to variation in humidity (Salisbury 1928; Torre *et al.* 2003), temperature (Ciha and Brun 1975; Luomala *et al.* 2005; Zhu *et al.* 2012) and carbon dioxide concentration (Woodward 1986), it is unknown how the balance between stomata and veins is maintained under such conditions.

While leaf anatomy (vein density and stomatal density/ size) determines conductances to liquid water and water vapour, it is the leaf environment that translates these conductances into water fluxes. In particular, atmospheric humidity expressed as the ambient leaf to air vapour pressure difference (VPD) determines

how much water is transpired at any given stomatal conductance. Thus, we would expect a leaf growing under high VPD conditions to require either a higher vein density or lower stomatal density than a leaf growing under low VPD conditions to maintain the same leaf water potential during midday transpiration. Published evidence of these responses, however, is somewhat contradictory. Studies of a range of herbs and trees have shown an increase in stomatal density with increasing VPD (Salisbury 1928; Leuschner 2002; Lake and Woodward 2008; Hovenden *et al.* 2012). However, the opposite response has been observed in tomato, capsicum, eggplant and *Rosa* (Bakker 1991; Torre et al. 2003). Furthermore, in the case of *Rosa*, the decrease in stomatal density was associated with an increase in vein density (Torre *et al.* 2003).

Assuming that the most efficient investment in stomata and veins occurs when stomata are able to open near to maximum aperture in hydrated soil and under saturated light (Franks *et al.* 2012), we would expect that increased VPD would induce a decrease in stomatal density/size or an increase in vein density. Thus, evaporative demand could be reduced to match water supply by reducing stomatal density or size, or alternatively vein density could be increased to improve hydraulic capacity, thus accommodating higher maximum transpiration. Therefore, we hypothesize that an increase in VPD would be associated with a commensurate change in the balance between vein and stomatal density.

In a previous study, we found that changes in epidermal cell size in response to differential leaf expansion under high and low irradiance allowed for coordinated development of leaf vein and stomatal density in the subtropical rainforest tree *Toona ciliata* M. Roem. (Carins Murphy *et al.* 2012). Leaves grown under high irradiance were significantly smaller and had higher vein and stomatal density than leaves grown under low irradiance (in which densities of veins and stomata were effectively ‘diluted’ by increased epidermal cell size). However, changes in VPD affect the transpiration rate per stomatal pore, potentially disrupting the balance

between liquid- and vapour-phase conductances in the leaf. Thus, if vein and stomatal densities remain proportional under increased VPD, a reduction in stomatal aperture would be required to maintain the balance between water conductances. However, the costs associated with having closed stomata mean that the most efficient use of resources would occur if anatomical adjustments (i.e. a reduction in stomatal density or an increase in vein density) mitigated the need for stomatal closure. Here, we compare plasticity in the density of stomata and veins of *T. ciliata* individuals grown under contrasting VPD to those observed under different light intensities to test the aforementioned hypothesis that acclimation to VPD would involve modification of the relationship between vein and stomatal density.

2.3 Materials and methods

2.3.1 Plant material

The subtropical woody angiosperm *T. ciliata* was selected for this experiment because it is a long-lived pioneer species capable of growing under a range of conditions (Herwitz *et al.* 1998) with known plasticity in leaf size, vein density and stomatal density (Carins Murphy *et al.* 2012). Four *T. ciliata* plants per VPD treatment were grown from commercially available seed (Seedlot 20189; Australian Tree Seed Centre, CSIRO, Highett, Vic., Australia) in a mixture of 76 % composted pine bark and 24 % coarse potting sand with fertilizer added. Plants were ~ 3 years of age and 1 m in height at the time of the experiment.

2.3.2 Growth conditions

All plants were transferred to a growth cabinet and grown under controlled conditions for 3 months, allowing a new cohort of leaves to initiate and expand. It was assumed that any new growth would respond to the conditions experienced during its development (Schoch *et al.* 1980). Growth conditions were 16 h days at 25

°C/20 °C day/night temperatures, with a maximum quantum photosynthetic photon flux density (PPFD) of 1000 µmol m^{-2} s^{-1}. Light was provided by a mixture of fluorescent and incandescent lights. A high VPD treatment was maintained at approximately 15 – 20 % relative humidity (or 2.5 – 2.7 kPa VPD) during the day by a commercial dehumidifier (Secco Ultra; Olimpia-Splendid, Gualtieri, Italy) and a vaporizer (TAAV Vaporaire steam vaporizer; Xidex Investments Pty. Ltd, Silverwater, NSW, Australia) maintained a low VPD treatment at approximately 70 – 80 % relative humidity (or 0.6 – 1 kPa VPD) during the day. Temperature and humidity were monitored for the duration of the experiment using a HOBO Pro RH/ Temp logger (Onset Computer Corporation Inc., Bourne, MA, USA).

2.3.3 Leaf gas exchange

Two leaves from each plant were used to determine the response of leaf gas exchange to variation in VPD. A portable infrared gas analyser (Li-6400; Li-Cor Biosciences, Lincoln, NE, USA) was used to measure stomatal conductance (mol m^{-2} s^{-1}), photosynthetic rate (µmol m^{-2} s^{-1}) and transpiration rate (mmol m^{-2} s^{-1}) between 1000 and 1300 h, when gas exchange was expected to be maximal. All variables within the leaf chamber of the Li-6400 were standardized during measurements (leaf temperature at 22 °C, CO_2 concentration between 380 and 390 µmol mol^{-1}; PPFD at 1000 µmol m^{-2} s^{-1}, and VPD at approximately 2.0 kPa for the high VPD treatment and 1.0 kPa for the low VPD treatment). Two leaves per plant grown under low VPD were also measured at 2.0 kPa to observe the dynamic response of stomata to high VPD. The midday water potential (−MPa) of all leaves was measured using a pressure chamber.

2.3.4 Leaf hydraulic conductance

The same two leaves per plant were used to determine leaf hydraulic conductance (mmol m^{-2} s^{-1} MPa^{-1}) under high and low VPD. Leaves were measured between 1000

and 1300 h using the evaporative flux method (Sack *et al.* 2002; Brodribb and Holbrook 2006). Leaves were excised and then immediately re-cut under water and attached to a flow meter (Brodribb and Holbrook 2006) (for construction details, see http://prometheuswiki.publish.csiro.au/tiki-index.php?page=Constructing+and+operating+a+hydraulics+flow+meter). Leaves were then placed in conditions favourable to transpiration (i.e. under a light source providing a PPFD greater than 200 µmol m^{-2} s^{-1} and heated evenly by a stream of warm air maintaining leaf temperature between 25 and 30 °C). Leaves were allowed to reach a transpirational steady state (less than 10 % variation over 180 s) and the resulting transpirational flux was recorded. Leaf water potential was measured with a pressure chamber and leaf hydraulic conductance calculated using the following equation:

$$K_L = F/\Psi_L,$$

where K_L is the leaf hydraulic conductance, F is the transpirational flux and Ψ_L is the leaf water potential at steady state. Leaf size was measured using a flatbed scanner in combination with ImageJ (National Institutes of Health, Bethesda, MD, USA). Leaf hydraulic conductance was normalized to leaf size and the viscosity of water at 20 °C using an empirical function based on data from Korson *et al.* (1969).

2.3.5 Anatomical traits

Vein density (total minor vein length per unit area) was determined from paradermal sections of each of the leaves used in gas exchange and leaf hydraulic conductance measurements. Sections were prepared and measured following the protocols described by Carins Murphy *et al*. (2012). In brief, this involved removing the adaxial epidermis and palisade tissue, clearing all pigment with bleach, and measuring vein density from slide mounts of these sections (two sections per leaf)

using image analysis of digital photomicrographs (five fields of view per section). Stomatal density (total stomata per unit area), stomatal size (mm^2) and epidermal cell size (mm^2) were also determined from cuticles (two per leaf and five fields of view per cuticle) prepared and measured following the protocols of Carins Murphy *et al*. (2012). Total vein length, number of stomata and number of epidermal cells per leaf were quantified by multiplying the density of veins, stomata and epidermal cells by leaf size.

2.3.6 Statistical analysis

The plasticity of leaf size, anatomical traits (vein density, stomatal density, epidermal cell size, stomatal size and total number of epidermal cells per leaf) and physiological traits (photosynthetic rate, stomatal conductance, transpiration rate, leaf hydraulic conductance and midday leaf water potential) were assessed by comparing the relative changes between plants grown under high and low VPD with unpaired *t*-tests. Dynamic changes to the stomatal conductance of plants grown under low VPD in response to short term exposure to high VPD were also assessed by comparing initial and final stomatal conductance using a paired *t*-test and by comparing final stomatal conductance with that of plants grown under high VPD using an unpaired *t*-test.

To test whether variation in vein and stomatal density between plants was passively determined by leaf size, vein and stomatal density were plotted against 1/√leaf size and 1/leaf size, respectively. The coordination between vein and stomatal density and leaf size traits was then quantified as the deviation from a proportional relationship (i.e. one in which vein and stomatal density increase uniformly with leaf expansion). The relationships between vein density and stomatal density and between anatomical traits (total epidermal cells per leaf, total stomata per leaf and total vein length per leaf) and leaf size were tested for proportionality in the same way.

ANCOVA analysis carried out using the R programming environment (R Core Team 2012) was used to test if the slope of the relationship between vein and stomatal density was the same regardless of whether changes were induced by VPD or by irradiance. r^2 was also calculated for both relationships.

The relationship between transpiration and leaf hydraulic conductance under high and low VPD was plotted against the same relationship in plants grown under high and low irradiance to test whether transpiration was maintained at rates appropriate for the hydraulic capacity of the vascular system. r^2 was calculated for the relationship between plants grown under contrasting irradiance.

2.4 Results

2.4.1 Response of leaf size and anatomy to variation in VPD

In *T. ciliata*, VPD had a substantial effect on leaf size and a small but significant effect on vein and stomatal density (Fig. 2.1). Leaves from plants grown under high VPD were about one-third (38 %) the size of those grown under low VPD ($P < 0.001$). However, leaves grown under high VPD had only 12 % more densely packed veins and 33 % more densely packed stomata ($P < 0.05$ in both cases) than those grown under low VPD. This response was considerably less than the proportional response expected if vein and stomatal density were controlled by the 'dilution' effect of leaf size ($P < 0.001$ and $P < 0.01$) (Fig. 2.2).

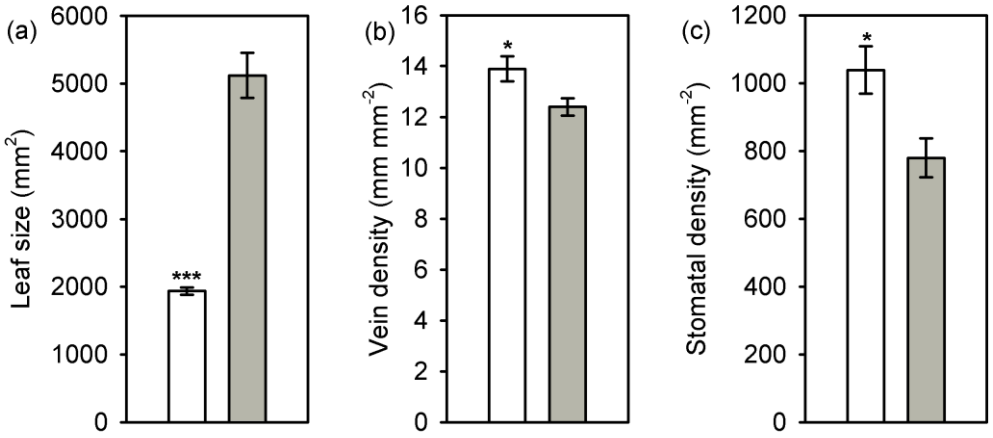

Figure 2.1 (a) Leaf size, **(b)** vein density and **(c)** stomatal density (± standard error) of *Toona ciliata* grown under high vapour pressure difference (VPD) (white columns) and low VPD (grey columns). Leaf size varied significantly between high and low VPD (***, $P < 0.001$) as did vein and stomatal density (*, $P < 0.05$).

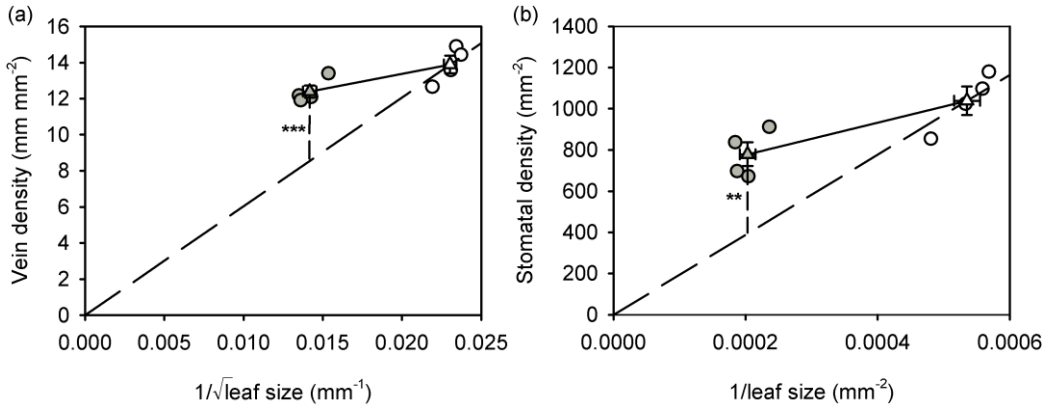

Figure 2.2 (a) Vein density and 1/√leaf size, and **(b)** stomatal density and 1/leaf size of *Toona ciliata* grown under high vapour pressure difference (VPD) (white symbols) and low VPD (grey symbols). Triangles indicate treatment means (± standard error); circles indicate values for individual plants. The slope of the mean response (solid line) was significantly different from the expected proportional relationship (when vein and stomatal density increase uniformly with leaf size) (broken line) in both cases (***, $P < 0.001$; **, $P < 0.01$).

Despite this, vein and stomatal density remained almost proportional across high and low VPD, although plants grown under low VPD had 20 % higher vein density than would be expected if vein and stomatal density were directly proportional ($P < 0.05$). Furthermore, the relationships between vein and stomatal density produced by changing VPD (this study) or irradiance (previous study) were not significantly different ($P > 0.05$) (irradiance data from Carins Murphy *et al*. (2012)) (Fig. 2.3).

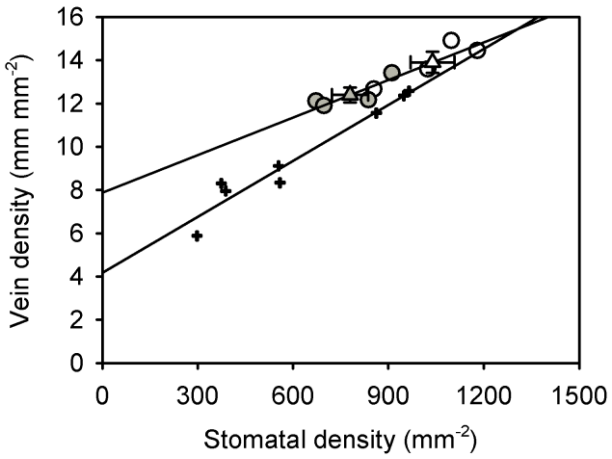

Figure 2.3 Vein and stomatal density of *Toona ciliata* grown under high vapour pressure difference (VPD) (white symbols) and low VPD (grey symbols) ($r^2 = 0.88$). Triangles are means (± standard error) and circles are values for individual plants. Also shown is the vein and stomatal density of *T. ciliata* grown under high and low irradiance (crosses) ($r^2 = 0.95$) (irradiance data from Carins Murphy *et al*. (2012)). Relationship slopes did not vary significantly between VPD and irradiance treatments ($P > 0.05$).

Epidermal cell size did not vary significantly between plants grown under high and low VPD ($P > 0.05$), although the stomata of plants grown under low VPD were slightly (14 %) larger than those of plants grown under high VPD ($P < 0.01$; Table 2.1). In contrast to this lack of effect on cell size, the total number of epidermal cells per leaf varied more than twofold between treatments so that the total number of epidermal cells per leaf was proportional to leaf size (Fig. 2.4). The total number of stomata and length of veins per leaf were also related to leaf size, although these

relationships were not directly proportional, as the total vein length per leaf in plants grown under low VPD was 9 % less than expected if directly proportional to leaf size, and the total number of stomata was 24 % less than would be expected if directly proportional to leaf size ($P < 0.05$ in both cases, data not shown).

Table 2.1 Comparison of anatomical and physiological traits between *Toona ciliata* plants grown under high and low vapour pressure difference (VPD) (values are means ± standard error)

Trait	Units	High VPD	Low VPD	*P*
S_{EC}	mm^2	$2.19 \times 10^{-4} \pm 1.71 \times 10^{-5}$	$2.66 \times 10^{-4} \pm 2.19 \times 10^{-5}$	0.146
S_S	mm^2	$1.29 \times 10^{-4} \pm 3.00 \times 10^{-6}$	$1.47 \times 10^{-4} \pm 2.69 \times 10^{-6}$	**0.005**
g_s	mol m^{-2} s^{-1}	0.09 ± 0.01	0.27 ± 0.03	**0.001**
K_L	mmol m^{-2} s^{-1} MPa^{-1}	10.15 ± 0.39	11.07 ± 0.94	0.397
A	µmol m^{-2} s^{-1}	8.47 ± 0.43	7.97 ± 0.61	0.535
E	mmol m^{-2} s^{-1}	1.65 ± 0.28	1.87 ± 0.12	0.485
Ψ_L	-MPa	0.82 ± 0.09	0.79 ± 0.02	0.810

Means of the two treatment groups were compared using unpaired *t*-tests. *P*-values < 0.05 are shown in bold.

S_{EC}, epidermal cell size; S_S, stomatal size; g_s, stomatal conductance; K_L, leaf hydraulic conductance; A, photosynthetic rate; E, transpiration rate; Ψ_L, midday leaf water potential.

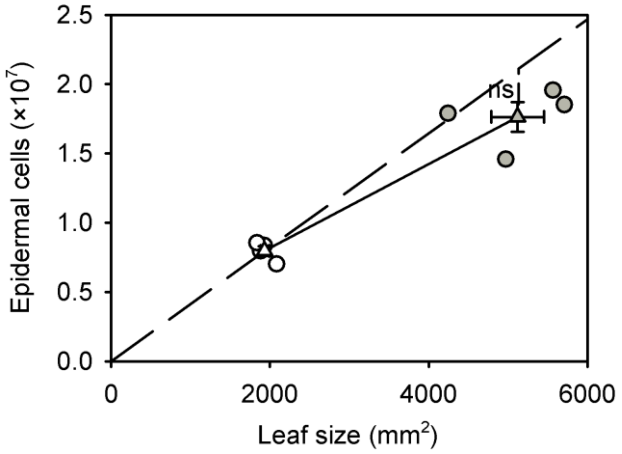

Figure 2.4 Total number of epidermal cells per leaf and leaf size of *Toona ciliata* grown under high vapour pressure difference (VPD) (white symbols) and low VPD (grey symbols). Triangles indicate means (± standard error); circles indicate values for individual plants. The total number of epidermal cells per leaf changed in proportion to leaf size so that the slope of the mean response (solid line) was not significantly different from the expected proportional relationship (when the total number of epidermal cells per leaf increases uniformly with leaf size) (broken line) (ns, $P > 0.05$).

2.4.2 Response of gas exchange, leaf hydraulic conductance and midday leaf water potential to high and low VPD

VPD had a large and significant effect on stomatal conductance. Plants grown under low VPD had 215 % higher stomatal conductance than those grown under high VPD ($P < 0.001$) (Table 2.1). However, variation in VPD did not induce a significant response in photosynthesis, transpiration, leaf hydraulic conductance or midday leaf water potential when measured under growth conditions ($P > 0.05$; Table 2.1) and transpiration was within the range of what would be predicted from the relationship between transpiration and leaf hydraulic conductance for this species under contrasting irradiance (Carins Murphy *et al*. 2012) (Fig. 2.5). Short-term exposure of plants grown under low VPD to high VPD conditions in the Li-Cor leaf chamber induced a 78 % reduction in stomatal conductance ($P < 0.01$), so that the final

stomatal conductance was not significantly different from that of plants grown under high VPD ($P > 0.05$).

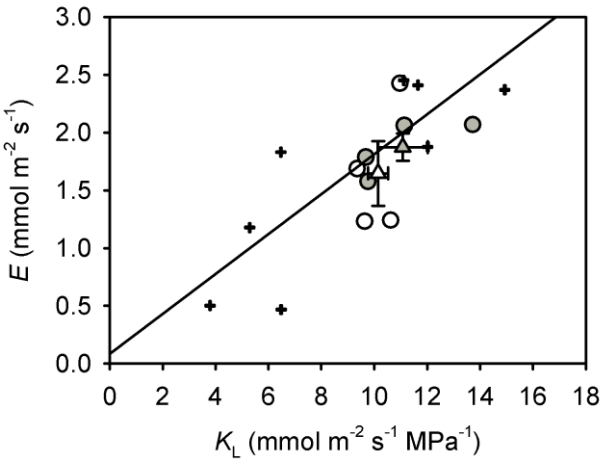

Figure 2.5 Transpiration (E) and leaf hydraulic conductance (K_L) of *Toona ciliata* grown under high vapour pressure difference (VPD) (white symbols) and low VPD (grey symbols). Triangles indicate means (± standard error); circles indicate values for individual plants. Also shown is the transpiration and leaf hydraulic conductance of *T. ciliata* grown under high and low irradiance (crosses) ($r^2 = 0.69$) (irradiance data from Carins Murphy *et al*. (2012)).

2.5 Discussion

2.5.1 Modification of stomatal conductance upholds transpirational homeostasis under high and low VPD

It was hypothesized that *T. ciliata* would accommodate the increased transpiration from stomatal pores at high VPD by changing the relationship between vein and stomatal density in the leaf. Contrary to this prediction, any change in this relationship was small, non-significant and in the opposite of the expected direction. Instead, we observed that the reduction in stomatal conductance necessary to maintain homeostasis was produced mainly by dynamic closure of stomata rather than a reduction in density. The small increase in stomatal density at high VPD was

coordinated with a similarly small increase in vein density. Thus, *T. ciliata* appears to be incapable of sufficient flexibility in the rates of initiation of veins and stomata to compensate for the different transpirational demands under high and low VPD. Instead, this species adjusts stomatal conductance to maintain transpiration (and leaf water potential) at a static level that is constrained by the density of veins (hydraulic supply efficiency). The effectiveness of dynamic stomatal closure in response to VPD is apparent from the lack of variation in transpiration, photosynthesis and midday leaf water potential between *T. ciliata* plants grown under high and low VPD. However, the capacity of *T. ciliata* to modify the relationship between vein and stomatal density would be further clarified by investigating the developmental response to low irradiance under contrasting VPD.

2.5.2 Vein and stomatal allocation is not optimized under high and low VPD

The relationship between vein and stomata density under different VPD levels in *T. ciliata* was similar to that observed across different irradiance levels (Fig. 2.3). Plants grown under the same VPD but different levels of irradiance adjust vein and stomatal density so that water supply and transpirational demand remain proportional (Carins Murphy *et al.* 2012). However, if plants grown under the same light intensity but different VPD used the same strategy, water loss would increase in proportion to water transport capacity in those plants grown under high VPD and stomata would close in response to declining leaf water potential. If it is assumed that the most efficient stomatal density occurs when stomata are able to open to near maximum aperture in fully hydrated soil (Franks *et al.* 2012), then the only way to optimally adjust leaf anatomy to variation in VPD is to alter the coordination between vein and stomatal density. Apparently, *T. ciliata* is not capable of making sufficient independent modification of vein and stomatal traits to compensate for large differences in VPD, meaning that leaves grown under high VPD are forced to operate with stomata mostly closed.

As with plants grown under high and low irradiance, changes in VPD induced large differences in leaf size, so that leaves were much larger in plants grown under low VPD. Changes in vein and stomatal density, however, were largely independent of leaf size. This is in contrast to the veins and stomata of plants grown under high and low irradiance that are passively 'diluted' or 'concentrated' by differential leaf expansion (Carins Murphy *et al.* 2012). Despite this, veins and stomata were still proportional across high and low VPD treatments. This suggests that the coordination of vein and stomatal density was not maintained by passive 'dilution' during leaf expansion but by coordinated initiation and differentiation of vein and stomatal cells.

The large effects of VPD on the number of epidermal cells and minimal effect on cell size resulted in the total number of epidermal cells per leaf being proportional to leaf size. Thus, leaves expanded under high VPD were smaller mainly due to reductions in initiation rather than expansion of the epidermal cells. This contrasts with the effects of irradiance on leaf size, which was mainly the result of differential epidermal cell expansion and effectively regulated the density of veins and stomata in concert (Carins Murphy *et al.* 2012). However, the differences in vein and stomatal density between plants grown under high and low VPD were much less than would be predicted from the large differences in leaf size. This indicates profound differences in the way that leaves adapt hydraulic and stomatal configuration in response to VPD and irradiance (Fig. 2.6).

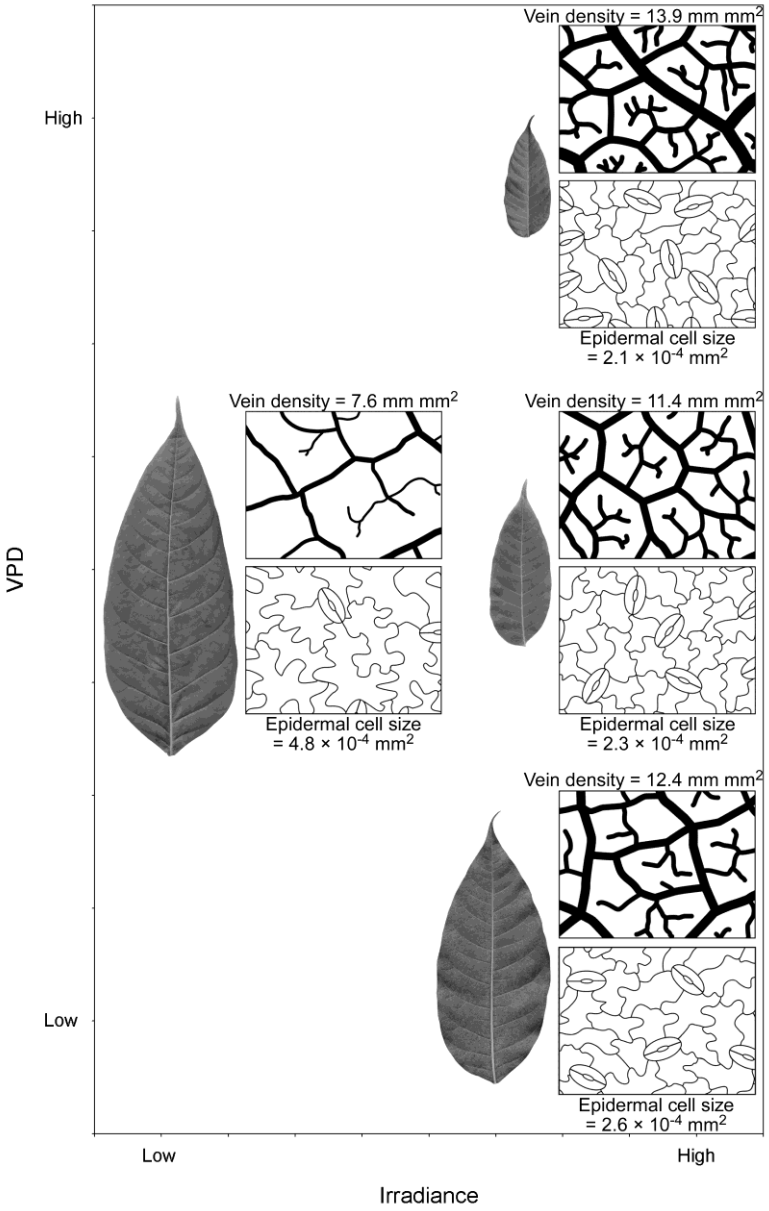

Figure 2.6 Changes to leaf size in response to differential irradiance and vapour pressure difference (VPD) in *Toona ciliata* are achieved by two contrasting methods. Leaf size is proportional to epidermal cell size in plants grown under high and low irradiance. This means that the comparatively greater leaf size typical of plants grown under low irradiance 'dilutes' the spacing of veins and stomatal on the leaf surface, while ensuring that vein and stomatal density remains proportional. In plants grown under high and low VPD, however, leaf size is proportional to the total number of epidermal cells per leaf. This method preserves high vein and stomatal density despite large changes to leaf size. Irradiance data from Carins Murphy *et al*. (2012).

2.5.3 Response of vein density, stomatal density and leaf size to VPD

The few studies that investigate the intraspecific response of vein and stomatal density to variation in VPD provide conflicting results. There is disagreement about the direction of the response and there is no discussion of the coordination (or lack of coordination) of veins and stomata under varying VPD. There are, however, fairly well-documented global trends across species of increasing leaf size with mean annual temperature and rainfall (conditions congruent with decreasing VPD) (Webb 1968; Wilf *et al.* 1998). Glasshouse trials also report increased growth rates and leaf size within species with decreasing VPD (Mortensen 1986; Mortensen 2000; Hovenden *et al.* 2012). As vein and stomatal density have been observed to increase with decreasing leaf size in some cases (specifically when smaller leaf sizes are induced by high irradiance) (Carins Murphy *et al.* 2012), it might be expected that greater VPD may also increase vein and stomatal density. However, in this study anatomical traits were independent of leaf size in *T. ciliata* grown under high and low VPD with only a weak trend towards higher vein and stomatal density in the smaller leaves produced under high VPD. This suggests that under varying VPD (but constant irradiance), vein and stomatal density are regulated by fixed ratios of cell production between veins and stomata, and that the plant modifies the number of cells in the leaf to produce large differences in leaf size. This is supported by evidence that differences in leaf size between *Nothofagus cunninghamii* plants grown under high and low humidity are related to the number of epidermal cells rather than the size of epidermal cells (Hovenden *et al.* 2012). Furthermore, in a separate study encompassing almost 500 dicotyledonous species, total vein density was found to be independent of leaf size (Sack *et al.* 2012), while another study found that variation in vein and stomatal density between leaves of the temperate rainforest tree *N. cunninghamii* grown under high and low irradiance was only partially explained by differences in leaf size (Brodribb and Jordan 2011). Thus, active determination of vein and stomatal density by an environmental stimulus such as VPD rather than a passive response to leaf size may account for the independence of vein and stomatal density from leaf size observed in this and other studies.

2.5.4 Implications for vein and stomatal development

Epidermal cell size was not significantly affected by VPD in *T. ciliata*. However, the total number of epidermal cells per leaf was greater in plants grown under low VPD. Increases in epidermal cell size are associated with water movement into growing cells (Cosgrove 1986), such that epidermal cell expansion in plants experiencing high transpiration rates is limited by competition for water (Fricke 2002). Epidermal cell division, on the other hand, is limited by photosynthetic input (Pantin *et al.* 2012). As epidermal cell size was not affected by VPD in *T. ciliata*, this implies that the water available for cell expansion was consistent across treatments. Thus, the homeostasis in transpiration observed in *T. ciliata* also brought about homeostasis in epidermal cell expansion. The total number of epidermal cells per leaf, however, varied with VPD so that plants grown under low VPD had more epidermal cells than those grown under high VPD. This difference suggests that epidermal cell initiation continued for longer in low VPD plants. There are several potential explanations for this: plants grown under low VPD may have been able to maintain higher net photosynthesis during the day (here, we only measured maximum assimilation in the morning), or it is possible that these plants invested less energy into roots in favour of leaf growth (Gislerød and Nelson 1989; Mortensen *et al.* 2001).They may also have experienced lower nocturnal transpiration rates, extending the time period during which cell division was possible.

Thus, differences in leaf size between plants grown under high and low VPD were the result of variation in epidermal cell division (not epidermal cell expansion) with plants grown under low VPD initiating more cells. This accounts for the disassociation of vein and stomatal density from leaf size. As veins and stomata are initiated during the early stages of leaf development before leaf expansion is completed (Pantin *et al.* 2012), increased initiation of epidermal cells would also result in increased initiation of veins and stomata (assuming a constant relationship between veins and stomata). The small differences in vein and stomatal density that

were observed between plants grown under high and low VPD may have been the result of a small (but not significant) variation in epidermal cell size.

2.6 Conclusions

Vein and stomatal density showed small but coordinated responses to growth under high and low VPD in *T. ciliata*, contradicting our hypothesis that the balance between vein and stomatal density would be modified to uphold transpirational homeostasis. Furthermore, the direction of this response was opposite to what was predicted (i.e. stomatal density increased with VPD). Instead, substantial changes in stomatal conductance maintained transpirational homeostasis in plants grown under contrasting VPD treatments. Thus, *T. ciliata* resorted to dynamic stomatal control rather than disruption of a fixed relationship between vein and stomatal tissue production. Consequently, the allocation of veins and stomata during leaf development must be tightly linked. Further research is needed to understand the developmental basis for this coordination.

Chapter 3

The coordination of veins and stomata in both herbs and woody species is dominated by cell expansion not cell differentiation

This chapter has been submitted to *Annals of Botany*:

Carins Murphy MR, Jordan GJ, Brodribb TJ (in review) The coordination of veins and stomata in both herbs and woody species is dominated by cell expansion not cell differentiation.

Chapter 4

Cell size is an important determinant of vein and stomatal density in ferns and angiosperms

4.1 Abstract

Vascular plant leaves are constructed so that water supply (leaf hydraulic conductance) matches both the maximum transpirational demand for water (stomatal conductance) under normal conditions and the maximum rate of photosynthetic assimilation. In angiosperms this balance is often achieved by a simple developmental mechanism in which changes to leaf cell size co-regulate vein and stomatal density (major determinants of water supply and demand, respectively). However, it is unknown how deep in the vascular plant phylogeny this 'passive dilution' mechanism is rooted. To investigate this we compared relationships between leaf minor vein density, stomatal density and epidermal cell size across four fern species with the same relationships across a diverse range of angiosperms from a previous study. Observed relationships across ferns were also compared with modelled relationships that assume vein and stomatal density respond passively to epidermal cell expansion and the plasticity of these traits was assessed in response to light intensity. Relationships between vein density, stomatal density and epidermal cell size across the ferns included in this study were remarkably similar to those previously observed across angiosperms. However, there was little plasticity in these traits within fern species and stomatal density was actively regulated by stomatal index as well as epidermal cell size. Despite this, epidermal cell size was an important determinant of vein and stomatal density in ferns (explaining 55.5 % of the variation in stomatal density versus 44.5 % explained by stomatal index). Thus, ferns (like angiosperms) appear to use the co-variance of vein and stomatal density with epidermal cell expansion to maintain a constant ratio between the abundance of veins and stomata in the leaf. This suggests the 'passive dilution' mechanism may be an ancient feature of vascular plants that co-regulates these tissues.

4.2 Introduction

Multiple lines of evidence suggest that the leaves of vascular plants are constructed so that the supply of water (leaf hydraulic conductance) matches both the maximum

transpirational demand for water (stomatal conductance) under normal conditions and the maximum rate of photosynthetic assimilation (Sack *et al.* 2003; Brodribb *et al.* 2005; Sack *et al.* 2005; Brodribb *et al.* 2007; Lo Gullo *et al.* 2010). Recent work suggests that this balance is often achieved in angiosperms developmentally by a simple mechanism in which changes to leaf cell size co-regulate vein density (total vein length per unit area) (a major determinant of water supply (Sack and Frole 2006; Brodribb *et al.* 2007)) and stomatal density (total number of stomata per unit area) (a major determinant of the demand for water) (Chapter 3; Carins Murphy *et al.* 2012; Brodribb *et al.* 2013). As vein synthesis is energetically costly for plants (Lambers and Poorter 1992) an oversupply of water results in unnecessary investment in vascular tissue (Chapter 3), while an undersupply restricts the uptake of carbon dioxide (CO_2) due to leaf dehydration. In other words, utilising cell expansion to maintain a constant ratio between vein and stomatal density ensures that investment in veins is enough that stomata are supplied with the minimum amount of water required to allow them to open to the optimal aperture set by leaf photosynthetic biochemistry (Medlyn *et al.* 2011). Under these circumstances plants are thought to optimise photosynthetic gain for the energy invested in construction and ongoing maintenance of leaf veins and stomata (Brodribb and Jordan 2011; Franks *et al.* 2012). However, it is not known when this 'passive dilution' mechanism appeared in the vascular plant phylogeny.

To investigate this we examined the relationships between vein density, stomatal density and epidermal cell size in early-branching vascular plants (ferns) and more recently evolved vascular plants (angiosperms). It is possible that ferns do not show proportional relationships between these traits because they differ from angiosperms in both current water relations physiology and evolutionary history. Consequently, ferns may retain some ancestral physiological characteristics that reflect the humid conditions during their early evolution in ancient, warm and relatively wet climates during the Palaeozoic and early Mesozoic (Page 2004; Sharpe *et al.* 2010). It is also likely that atmospheric CO_2 concentrations at this time were considerably higher than

those experienced during the diversification of the angiosperms (Berner 2006; Brodribb *et al.* 2009). Certainly, most ferns today are restricted to relatively mesic habitats and drought tolerant species are largely restricted to a few derived clades (Sharpe *et al.* 2010). In addition, ferns are unable to maintain homeostasis in water-use efficiency because they lack a number of key adaptations in stomatal function that allow angiosperms to respond actively to changes in the internal and external environment (Brodribb and McAdam 2011; McAdam and Brodribb 2012). Although responsive to light, fern stomata do not respond to the rate of CO_2 assimilation like angiosperms (Wong *et al.* 1979; McAdam and Brodribb 2012) and, instead, are very sensitive to leaf water status. Thus, ferns may have experienced different selective pressures to angiosperms during their early evolution and as a result may have different mechanisms to maximise the benefit-cost ratio between photosynthetic return and energetic investment in vein and stomatal infrastructure.

There is some evidence to suggest that ferns can adaptively regulate the ratio between vein and stomatal density (Zhang *et al.* 2014). However, it is not known whether these relationships are maintained by the co-variance of vein and stomatal density with epidermal cell expansion (the 'passive dilution' mechanism) as has been observed in angiosperms. Thus, this study aims to test whether the relationships between vein density, stomatal density and epidermal cell size across ferns are similar to the proportional relationships observed across angiosperms. To do this we compared four fern species from this study with a diverse range of angiosperm species from a previous study. We also compared relationships between vein density, stomatal density and epidermal cell size across ferns with modelled relationships that assume vein and stomatal density respond passively to epidermal cell expansion and assessed the plasticity of these traits in response to light intensity.

4.3 Materials and methods

4.3.1 Plant material

Four fern species were selected for the experiment based on the diversity of their preferred light habitats (Benham and Windham 1992; Hoshizaki and Wilson 1999; Parris 2001; McGlone *et al.* 2005) (Table 4.1). Three plants per species were grown in controlled conditions under either sun or shade in a mixture of 76 % composted pine bark and 24 % coarse potting sand and received weekly applications of liquid fertiliser (Aquasol, Hortico). Plants experienced day and night temperatures of 15 and 25 ºC, respectively, and ambient relative humidity. Natural light was supplemented by sodium vapour lamps in the morning and evening to maintain a 14 hour photoperiod. Plants grown in the sun treatment received a maximum photosynthetic photon flux density (PPFD) of approximately 1800 µmol m^{-2} s^{-1} while plants in the shade treatment were grown under 90 % shade cloth and received a maximum PPFD of approximately 200 µmol m^{-2} s^{-1}. Data from these species were compared with data from nine angiosperm species from a previous study grown under similar controlled sun and shade conditions (Chapter 3).

Table 4.1 List of experimental species

Species	Family	Light habitat
Astrolepis sinuata (Lag. ex Sw.) D.M. Benham & Windham	Pteridaceae	Sun
Dryopteris cycadina (Franch. & Sav.) C. Chr.	Dryopteridaceae	Shade
Pteridium esculentum (G. Forst.) Cockayne	Dennstaedtiaceae	Both
Todea barbara T. Moore	Osmundaceae	Sun

4.3.2 Stomatal conductance

Two healthy, mature leaves (fronds) from the most recently expanded cohort were selected per plant (n = 3) to determine maximum stomatal conductance (mol m^{-2} s^{-1}) using a Li-Cor Biosciences Li-6400 portable infrared gas analyser (Lincoln, NE, USA). Measurements were performed between 1000 and 1300 h when rates were expected to be maximal. Leaf chamber conditions were standardised during measurements. Leaf temperature was maintained at 25 ºC, CO_2 concentration between 380 and 390 µmol mol^{-1}, vapour pressure difference (VPD) at approximately 1.3 kPa and PPFD at 1000 µmol m^{-2} s^{-1}.

4.3.3 Pinna size and anatomical traits

The leaves used to determine stomatal conductance were scanned at 300 pixels per inch (dpi) using a Canon CanoScan CS8800F flatbed scanner (Sydney, Australia) to measure pinna size (mm^2). Three pinna were measured per leaf using the image analysis software ImageJ (Rasband 1997-2014), and averaged to get the mean pinna size per leaf. Vein density (mm mm^{-2}), stomatal density (mm^{-2}) (all species were hypostomatic), stomatal size (mm^2), stomatal index, epidermal cell density (total number of epidermal cells per unit area; mm^{-2}) and epidermal cell size (mm^2) were then quantified using paradermal sections taken from two locations on three pinna from each leaf (i.e. six sections per leaf). To prepare the paradermal sections the adaxial epidermis and palisade tissue were removed with a sharp razor and the remaining tissue placed in commercial household bleach (50 g L^{-1} sodium hypochlorite and 13 g L^{-1} sodium hydroxide) until all pigment was removed. Sections were then rinsed, stained with 1 % toluidine blue and mounted on microscope slides in phenol glycerine jelly.

Entire sections were scanned at 1600 dpi to calculate vein density using ImageJ. This involved dividing the total length of all veins contained within a section by the

section area. To quantify all other anatomical traits five fields of view were photographed from all sections using a Nikon Digital Sight DS-L1 camera (Melville, NY, USA) mounted on a Leica DM 1000 microscope (Nussloch, Germany). Photomicrographs were taken of sections from the sun and shade leaves of *Dryopteris cycadina* and *Todea barbara* at 10 × objective magnification (field of view area 0.56 mm^2), from the sun and shade leaves of *Astrolepis sinuata* and the shade leaves of *Pteridium esculentum* at 20 × objective magnification (field of view area 0.14 mm^2) and from the sun leaves of *P. esculentum* at 20 × objective magnification through a 2.5 × tube (field of view area 0.025 mm^2). Additional photomicrographs were taken of sections from the sun leaves of *P. esculentum* at 40 × objective magnification through a 0.7 × tube (field of view area 0.073 mm^2) using differential interference contrast with a Leica DFC450 digital microscope camera mounted on a Leica DM 2000 LED microscope to overcome the partial obscuration of epidermal cells by trichomes.

All leaf anatomical traits were quantified using ImageJ. Stomatal size was measured from five stomata (comprising a pair of guard cells) per field of view. Epidermal cell size (S_{EC}) was calculated as:

$$S_{EC} = ((1 - (D_S \times S_S)) / D_{EC}, \quad (4.1)$$

where D_S is stomatal density, S_S is stomatal size and D_{EC} is epidermal cell density. Stomatal index (SI) was calculated as:

$$SI = (D_S / (D_S + D_{EC})) \times 100, \quad (4.2)$$

according to Salisbury (1928). Partial stomata and epidermal cells were included in density counts if visible along the top and right-hand border of photomicrographs and discarded if visible along the bottom and left-hand border.

4.3.4 Statistical analysis

The plasticity of vein density, stomatal density, stomatal index, pinna size, epidermal cell size and stomatal conductance in response to light intensity was assessed by comparing relative changes between plants grown under sun and shade with unpaired *t*-tests. ANOVA performed in R (R Core Team 2014) was then used to test if a single linear regression could describe the relationship between vein density and √stomatal density across all ferns and a similar data set of nine angiosperm species from a previous study. The same approach was used to compare the relationships between vein density and 1/√epidermal cell size and between stomatal density and 1/epidermal cell size across all ferns from this study and the nine angiosperm species. The correlation coefficient (r^2) and statistical significance ($P < 0.05$) of the co-variation between stomatal density and 1/epidermal cell size was then calculated incorporating all ferns and angiosperms. Epidermal cell size and stomatal density were transformed according to the expectation that cell expansion dictates vein and stomatal density.

The correlation coefficient and statistical significance of the co-variation between vein density and √stomatal density, vein density and 1/√epidermal cell size, stomatal density and 1/epidermal cell size, stomatal density and stomatal index, and stomatal conductance and vein density across all four fern species was then determined using R. The relative contribution of 1/epidermal cell size and stomatal index to the r^2 of the multiple regression in which they are predictors of stomatal density was quantified as a relative importance metric (lmg) using the 'relimp' package in R.

4.3.5 'Passive dilution' models

Relationships between vein density, stomatal density and epidermal cell size across all fern species were compared with modelled relationships using ANOVA performed in R. Modelled relationships were calculated according to the method outlined in Chapter 3. Hence, they were based on the 'passive dilution' hypothesis whereby vein and stomatal density are coordinated by the expansion of epidermal cells. This assumes that vein and stomatal densities are uniquely related to epidermal cell size and that the epidermis comprises only epidermal and stomatal cells with a constant ratio between them (stomatal index). Thus, epidermal cell size was calculated for a range of stomatal densities using equation 4.1 where epidermal cell density was calculated as:

$$D_{EC} = (D_S / SI) - D_S, \quad (4.3)$$

using three different stomatal indices (the mean across all fern species and the mean ± 20 %) and the mean stomata size across all ferns species (there was no significant relationship between stomatal density and size among the ferns (Fig. 4.1)).

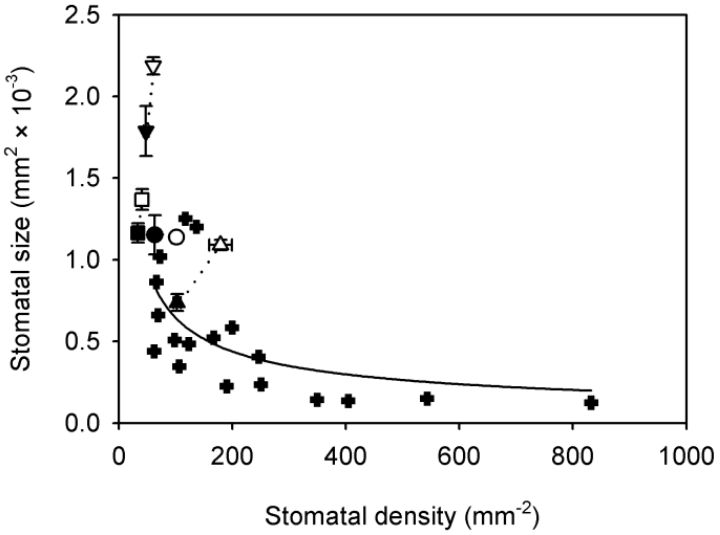

Figure 4.1 Mean stomatal size and density ± standard deviation of four fern species *Astrolepis sinuata* (circles), *Dryopteris cycadina* (squares), *Pteridium esculentum* (up-facing triangles) and *Todea barbara* (down-facing triangles) grown in sun (open symbols) and shade (closed symbols) (species pairs joined by a dotted black line). Also shown is the relationship between stomatal size and density across nine angiosperm species (black crosses) (data from Chapter 3). There was no significant relationship across the ferns. Solid black line is the best fit regression across the angiosperms (r^2 = 0.37, P < 0.01).

The relationship between vein density and epidermal cell size was modelled using the simplified assumption that vein length is associated with a fixed proportion of the perimeter of an epidermal cell. According to this assumption one side of a theoretical square epidermal cell would always be in contact with vein tissue as it expanded. Thus, a geometric model of vein density as a function of epidermal cell size was determined for a fixed stomatal index (the mean across fern species). Epidermal cell size was calculated as above. Assuming vein density (D_V) was a function of epidermal cell size we fitted the function:

$$D_V = a \times S_{EC}^{-0.5}, \quad (4.4)$$

where *a* is proportional to the epidermal cell perimeter associated with vein length. This returned a value of 0.1071 for *a*. This value was then used to predict the impact of epidermal cell size on vein density (using the equation: $D_V = 0.1071 \times S_{EC}^{-0.5}$).

4.4 Results

4.4.1 Response of pinna size and leaf anatomy to sun and shade

The magnitude and direction of change in vein and stomatal density in response to sun and shade varied between the four fern species (Table 4.2). Shade induced a significant decrease in vein density in *P. esculentum*, a significant increase in *D. cycadina*, and had no significant effect on vein density in *A. sinuata* or *T. barbara*. Shade induced significant decreases in stomatal density in all species except *D. cycadina* in which there was no significant response of stomatal density to irradiance. Stomatal index was significantly lower in the shade leaves of all species except *A. sinuata* (Table 4.2). There was no significant response of pinna size or epidermal cell size to irradiance, except in *A. sinuata* where epidermal cells were significantly larger in shade leaves than in sun leaves (Table 4.2).

Table 4.2 Percentage decrease in leaf traits from sun to shade

Species	Vein density	Stomatal density	Stomatal index	Pinna size	Epidermal cell size
Astrolepis sinuata	10.3 ns	37.8 **	15.2 ns	2.1 ns	-42.6 *
Dryopteris cycadina	-21.8 **	17.7 ns	16.1 *	24.3 ns	-10.2 ns
Pteridium esculentum	21.1 **	42.6 **	35.1 **	-28.5 ns	-34.1 ns
Todea barbara	-10.6 ns	20.9 **	34.5 **	14.3 ns	4.7 ns

Symbols indicate a significant difference between sun and shade plants (results of unpaired *t*-tests) (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, $P > 0.05$).

4.4.2 Comparisons with angiosperm data

The slope of the relationship between vein density and √stomatal density across all fern species was not significantly different from the slope of the same relationship across nine angiosperm species ($P > 0.05$) (Fig. 4.2). However, the intercept of the relationship across the ferns was significantly different from the intercept of the relationship across the angiosperms ($P < 0.05$). Likewise, the relationship between vein density and 1/√epidermal cell size across all fern species had a similar slope to the same relationship across the angiosperm species ($P > 0.05$), but the intercept was significantly different ($P < 0.001$) (Fig. 4.3a). Thus, fern leaves had less vein length per stomata or epidermal cell than angiosperms leaves. In comparison, the slope and intercept of the relationship between stomatal density and 1/epidermal cell size across all fern species was not significantly different from the slope and intercept of the same relationship across the angiosperms ($P > 0.05$ in both cases) (Fig. 4.3b). Furthermore, the relationship between stomatal density and 1/epidermal cell size across all fern and angiosperm species was described by a highly significant linear regression ($r^2 = 0.91$; $P < 0.001$).

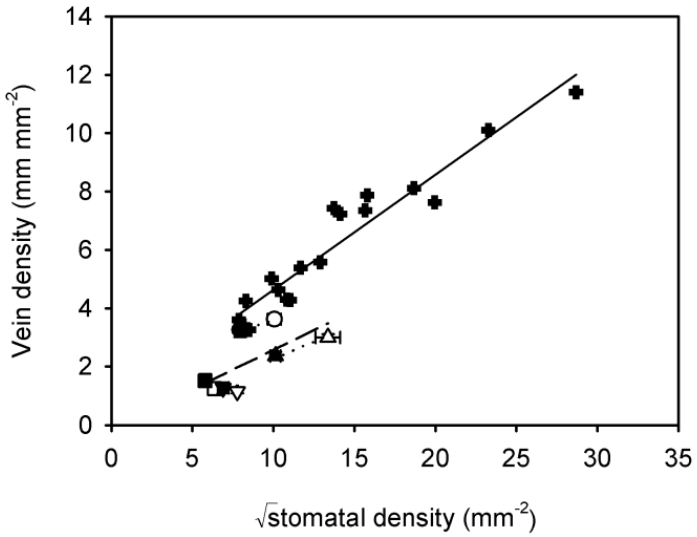

Figure 4.2 Relationship between vein and √stomatal density across all fern species (black dashed line) compared with the same relationship across nine angiosperm species (data from Chapter 3) (black crosses and black solid line). Fern values are means ± standard deviation (symbols as in Fig. 4.1).

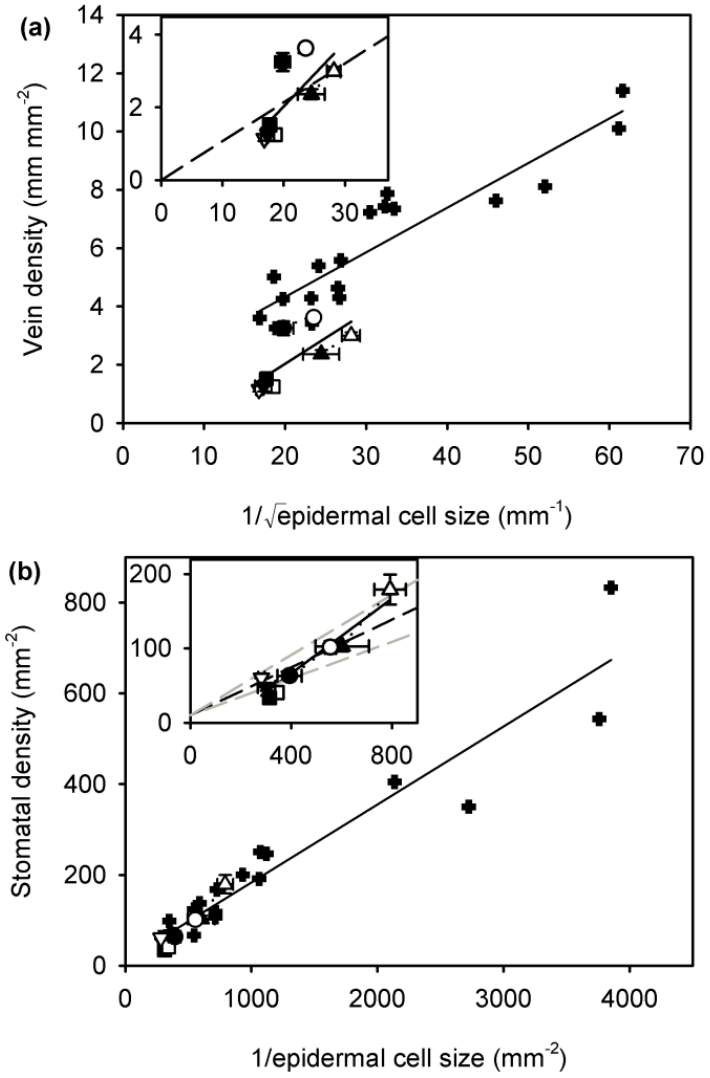

Figure 4.3 Relationships between **(a)** vein density and 1/√epidermal cell size and **(b)** stomatal density and 1/epidermal cell size across all ferns species (black solid lines) compared with the same relationships across nine angiosperm species (data from Chapter 3) (black crosses and black solid lines). Fern values are means ± standard deviation (symbols as in Fig. 4.1). A significant linear regression describes the relationship between stomatal density and 1/epidermal cell size across all fern and angiosperm species (D_S = 0.172 × 1/S_{EC} + 11.001; r^2 = 0.91; P < 0.001). Inserts show relationships between vein density and 1/√epidermal cell size and between stomatal density and 1/epidermal cell size across all ferns species grown under sun and shade (black solid lines) compared with modelled relationships (dashed lines). Modelled relationships assume that epidermal cell expansion drives vein and stomatal density and that stomatal index is constant. Black dashed lines are models using the fern mean stomatal index and grey dashed lines are

models using the fern mean stomatal index ± 20 % (see materials and methods for details). The slope of the observed relationship between vein density and 1/√epidermal cell size was not significantly different from the slope of the modelled relationship, while the slope of the observed relationship between stomatal density and 1/epidermal cell size was significantly different from the slope of the modelled relationship using the fern mean stomatal index. Significant linear regressions described the observed relationships between vein density and 1/√epidermal cell size (D_V = 0.177 × 1/√S_{EC} - 1.511; r^2 = 0.52; P < 0.05) and between stomatal density and 1/epidermal cell size (D_S = 0.252 × 1/S_{EC} - 34.335; r^2 = 0.92; P < 0.001) across the ferns.

4.4.3 Relationships between vein density, stomatal density, epidermal cell size and stomatal index across ferns

Vein density and √stomatal density were not significantly correlated across fern species (P > 0.05), although there was a trend in the expected direction across species and within *P. esculentum* and *A. sinuata* (Fig. 4.2). However, across all fern species vein density was significantly correlated with 1/√epidermal cell size (r^2 = 0.52; P < 0.05) (Fig. 4.3a), and stomatal density was strongly correlated with 1/epidermal cell size (r^2 = 0.92; P < 0.001) (Fig. 4.3b) and stomatal index (r^2 = 0.81; P < 0.01) (Fig. 4.4). The slope of the observed relationship between vein density and 1/√epidermal cell size was not significantly different from the slope of the modelled relationship (P > 0.05) (insert in Fig. 4.3a). However, the slope of the observed relationship between stomatal density and 1/epidermal cell size was 44.7 % greater than the slope of the modelled relationship (P < 0.01) (insert in Fig. 4.3b). Despite this, 1/epidermal cell size was a more important determinant of stomatal density than stomatal index (explaining 55.5 % versus 44.5 % of the variation).

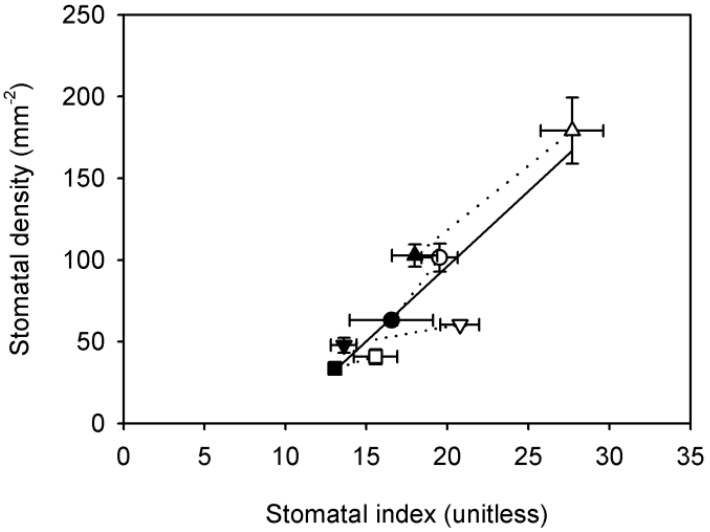

Figure 4.4 Relationship between stomatal density and stomatal index across all fern species. Values are means ± standard deviation (symbols as in Fig. 4.1). The relationship is described by a significant linear regression (D_S = 9.193 × SI - 87.626; r^2 = 0.81; P < 0.01).

4.4.4 Stomatal conductance and vein density

Rates of stomatal conductance were significantly lower in the shade leaves of three of the four ferns species than in sun leaves (56.7 % in *D. cycadina*; P < 0.01, 41.9 % in *P. esculentum*; P < 0.05 and 39.1 % in *T. barbara*; P < 0.01). Stomatal conductance was 26 % lower in the shade leaves of *A. sinuata* than in sun leaves but this change was not statistically significant (P > 0.05). Furthermore, there was a significant positive relationship between stomatal conductance and vein density across all fern species (r^2 = 0.56; P < 0.05; Fig. 4.5).

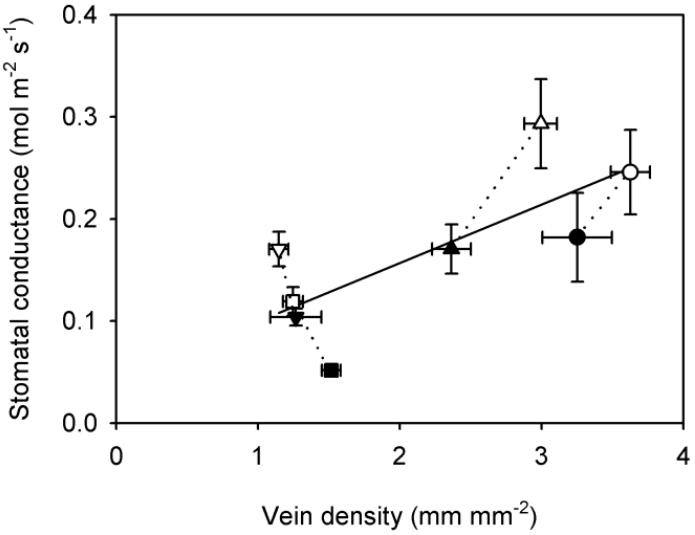

Figure 4.5 Mean stomatal conductance and vein density ± standard deviation of four fern species grown under sun and shade (symbols as in Fig. 4.1). A significant regression describes the relationship between stomatal conductance and vein density across all species (black solid line; $g_s = 0.0572 \times D_V + 0.0426$; $r^2 = 0.56$; $P < 0.05$).

4.5 Discussion

4.5.1 Proportional relationships between vein density, stomatal density and epidermal cell size are similar across ferns and angiosperms

Despite expectations that ferns may employ different mechanisms to angiosperms to maintain a constant ratio between vein and stomatal density, relationships between vein density, stomatal density and epidermal cell size across the fern species included in this study were remarkably similar to those previously observed across angiosperm species in Chapter 3. Notably, the relationship between stomatal density and epidermal cell size across fern species was not significantly different from the same relationship across the angiosperm species. Furthermore, the slopes of the relationships between vein and stomatal density and between vein density and epidermal cell size across fern species were not significantly different from the slopes of the same relationships across the angiosperms species. The intercepts of both relationships, however, were lower in the ferns indicating that they produce less

vein length per stomata or epidermal cell than angiosperms. A smaller investment in veins may be linked to the lower maximum stomatal porosity of ferns compared with angiosperms (Franks and Farquhar 2007). Despite the generally large size of fern stomata, small maximum apertures mean that ferns have intrinsically lower stomatal conductance than angiosperms for the same stomatal density. Thus, ferns appear to maintain a fairly constant ratio between vein and stomatal density by the same 'passive dilution' mechanism as angiosperms. These results suggest that the co-variance of vein and stomatal density with epidermal cell expansion may be an ancient mechanism that co-regulates these parameters.

4.5.2 Epidermal cell size is an important determinant of vein and stomatal density in ferns

Vein and stomatal density were not significantly correlated across the four fern species included in this study; however, there were trends in the expected direction across species and within *P. esculentum* and *A. sinuata*. The weakness of this relationship was most likely due to variation in how vein and stomatal density respond to epidermal cell size as well as the very small range in vein and stomatal density. Vein density was correlated with epidermal cell size, and the slope of this relationship was not significantly different from the slope predicted if vein density was passively 'diluted' by epidermal cell expansion. However, stomatal density was correlated with stomatal index as well as epidermal cell size. Consequently, the slope of the relationship between stomatal density and epidermal cell size was significantly different from the slope predicted if stomatal density were passively regulated by epidermal cell expansion. Thus, the ferns sampled here appeared to utilise epidermal cell expansion to mediate vein and stomatal density but not to the same extent as has been observed in angiosperm species. This was due to modification of the frequency of stomatal initiation (the stomatal index) that may have occurred independently of changes to vein density. Despite this, epidermal cell size was still an important

determinant of vein and stomatal density in ferns (explaining 55.5 % of the variation in stomatal density versus 44.5 % explained by stomatal index).

4.5.3 Other factors that may affect the coordination of vein and stomatal density

The plasticity of vein density, stomatal density and epidermal cell size was fairly limited within fern species in response to light intensity. In angiosperms, large changes to epidermal cell size are required to significantly adjust vein and stomatal density (Chapter 2; Carins Murphy *et al.* 2014). Hence, ferns may rely more heavily on active adjustment of stomatal density because they have a limited capacity to manipulate epidermal cell size. Not only would this potentially reduce the coordination between vein and stomatal density within species it may also place an upper limit on the rates of gas exchange achieved by ferns because they may be unable to reduce epidermal cell size enough to produce high densities of veins and stomata. This premise is supported by the correlation between vein density and stomatal conductance across the ferns included in this study and the low vein densities generally observed in ferns (Boyce *et al.* 2009). The weak relationship observed between stomatal density and size in the ferns studied here may also reduce the degree of coordination between veins and stomata. Together stomatal density and pore size determine maximum stomatal conductance and are generally well correlated across plant species (Franks and Beerling 2009). Thus, stomatal density may be only weakly related to stomatal conductance (and hence vein density) if stomatal pore size is modified in isolation.

4.5.4 Differences in leaf development may contribute to the reduced dependence of stomatal density on epidermal cell expansion in ferns compared with angiosperms

Ferns are less dependent on epidermal cell expansion to coordinate vein and stomatal density than angiosperms. This may be due to general differences in leaf development between clades. Fern leaves undergo marginal development which

generally results in bifurcating vascular systems that lack hierarchical vein orders (Pray 1960; Pray 1962; Zurakowski and Gifford 1988; Roth-Nebelsick *et al.* 2001), while angiosperms undergo diffuse leaf development and can produce reticulate vascular systems with many vein orders (Pray 1955a; Pray 1955b; Esau 1960; Poethig and Sussex 1985a; Poethig and Sussex 1985b). The strong link between vein density, stomatal density and epidermal cell size seen in angiosperms is conducive to most veins and stomata being initiated before the completion of leaf expansion (Pantin *et al.* 2012). In contrast, the partial independence of stomatal density from epidermal cell size observed in the ferns included in this study suggests that stomata initiation may continue for longer during leaf expansion. As well as potentially reducing the degree of coordination between vein and stomatal density in ferns, marginal development may also result in a less homogenous distribution of stomata throughout the leaf epidermis and a decrease in overall rates of CO_2 uptake.

4.6 Conclusions

Relationships between vein density, stomatal density and epidermal cell size across four ferns species were very similar to those observed across angiosperm species in a previous study. However, there was little plasticity in these parameters within ferns species and changes to stomatal density across species were actively regulated by stomatal index, as well as epidermal cell size. Despite this, epidermal cell size was a strong determinant of vein and stomatal density in ferns (explaining 55.5 % of the variation in stomatal density versus 44.5 % explained by stomatal index). Thus, ferns (like angiosperms) appear to use the co-variance of vein and stomatal density with epidermal cell expansion to maintain a constant ratio between the abundance of veins and stomata in the leaf. This suggests the ‘passive dilution’ mechanism may be an ancient feature of vascular plants that co-regulates these tissues.

Chapter 5

Vein density is independent of variation in epidermal cell size among *Arabidopsis* genotypes

5.1 Abstract

Coordination of vein and stomatal density has been observed within and across a range of vascular plants. This ensures there are sufficient veins for stomata to receive the minimum amount of water required to maintain an optimal aperture under well-watered conditions, thus, maximizing the benefit to cost ratio of leaf construction. It was recently proposed that coordination of these traits is achieved by a 'passive dilution' mechanism in which densities of veins and stomata are co-regulated by epidermal cell size. However, unlike stomata, veins are spatially isolated from the epidermis and it is not known whether vein density is directly regulated by epidermal cell expansion. To investigate this we tested whether relationships between vein density and epidermal cell size in a wild type genotype of *Arabidopsis* (Col-0) and seven other genotypes with modified forms of genes that affect both stomatal development and epidermal cell size were the same as modelled relationships that assume veins are passively 'diluted' by epidermal cell expansion. Vein density in wild type plants was correlated with abaxial epidermal cell size in a way that was consistent with the 'passive dilution' mechanism (despite some deviation from modelled relationships). However, vein density was independent of variation in epidermal cell size among mutant and transgenic genotypes. This suggests that epidermal cell size in these genotypes was modified independently from the rest of the leaf in spite of prior evidence that cell sizes are correlated within leaves. Thus, vein density is not causally linked to epidermal cell expansion. Instead the relationship between vein density and epidermal cell size in wild type plants reflects developmental factors that affect both mesophyll and epidermal cells, suggesting that adaptation favours coordination of veins and stomata over independent development of these tissues.

5.2 Introduction

The correlation between leaf conductances to liquid- and vapour-phase water across a diverse range of plants (Brodribb *et al.* 2005; Lo Gullo *et al.* 2010) suggests that a balance between water supply and transpirational demand is established during leaf construction. The efficiency with which water is transported through the leaf mesophyll to supply evaporative surfaces near the stomata is determined by leaf vein density (total vein length per unit area) (Sack and Frole 2006; Brodribb *et al.* 2007) while transpirational demand is determined by stomatal density (total number of stomata per unit area) and aperture. Thus, if there is an imbalance between the production of veins and stomata during leaf development the plant suffers diminishing photosynthetic returns on energetic investments in vein construction (Lambers and Poorter 1992) or stomatal maintenance. Thus, the maximum benefit to cost ratio is expected when investment in the leaf vein network is sufficient that stomata are supplied with the minimum amount of water required to allow them to open to the optimal aperture set by leaf photosynthetic biochemistry (Medlyn *et al.* 2011) under well-watered conditions (Brodribb and Jordan 2011; Franks *et al.* 2012). This view is supported by evidence for developmental coordination of veins and stomata within individual plants, within species, across populations of the same species and across species in a range of woody and herbaceous angiosperms and ferns (Chapters 3 and 4; Brodribb and Jordan 2011; Carins Murphy *et al.* 2012; Zhang *et al.* 2012; Brodribb *et al.* 2013; Martins *et al.* 2014; Yang *et al.* 2014; Zhang *et al.* 2014; Fiorin *et al.* in press).

In Chapter 3 we proposed that vein and stomatal development is coordinated via a 'passive dilution' mechanism in which densities of veins and stomata are co-regulated by epidermal cell size. However, unlike stomata, veins are spatially isolated from the epidermis and it is not known whether they are directly regulated by differential epidermal cell expansion. Although there is evidence that, across a wide range of angiosperms, cell sizes of all major tissues types are correlated within leaves (Brodribb *et al.* 2013; John *et al.* 2013), and that these changes in cell size are

associated with changes in vein and stomatal density such that vein density is correlated with modelled demand for water (Brodribb *et al.* 2013). Despite this overall link, the size and abundance of different cell types can be under independent control. For example, the differentiation of procambial cells into vascular cells during leaf vein development is regulated by movement of the phytohormone auxin through developing leaf tissue (Jacobs 1952; Sachs 1981; Uggla *et al.* 1996; Sieburth 1999; Avsian-Kretchmer *et al.* 2002; Aloni *et al.* 2003). According to the 'auxin-flow canalisation hypothesis' cell polarity determines the direction of auxin transport, and sustained transport induces increased polarity creating feedback that leads to canalisation of auxin flow and cell differentiation into defined strands (Sachs 1981). Carrier proteins that are located at the basal end of cells regulate the polarity of auxin flow by mediating the transfer of auxin (Rubery and Sheldrake 1974; Raven 1975; Galweiler *et al.* 1998). In comparison, stomatal development occurs in a series of transitions from undifferentiated protodermal cells to meristemoid mother cells, meristemoids, guard mother cells and finally to mature stomata (Pant and Kidwai 1967). This process is tightly regulated by a complex signalling network involving an independent suite of genetic controls (Lau and Bergmann 2012; Pillitteri and Torii 2012). The prominent role of auxin in vascular development and evidence that mutations affecting auxin transport impact stomatal differentiation (Mayer *et al.* 1993; Spitzer *et al.* 2009) led to the suggestion that auxin may link vein and stomatal development (Brodribb and Jordan 2011). However, recent research has indicated that auxin is a negative regulator of stomatal development (Balcerowicz and Hoecker 2014). The positive relationship observed between vein and stomatal density is therefore unlikely to involve auxin signalling as the high concentrations of auxin required to produce high vein densities would likely inhibit stomatal differentiation.

Thus, we aimed to investigate whether the developmental control of veins and epidermal tissues is linked (consistent with the 'passive dilution' mechanism), and if this affects the coordination of water supply and demand. Recent advances in the understanding of stomatal development have produced a suite of genotypes that

specifically alter stomatal density, stomatal index, and epidermal cell size, which consequentially alter the gas exchange capacity of the leaf (Dow and Bergmann 2014). Isolated control of cellular development in the epidermis allows us to observe whether changes in external tissues remain coordinated with developmental programs in adjacent and physiologically linked internal tissues. We predicted that stomatal density would be regulated by genetic changes to stomatal index and, more generally, to epidermal cell expansion, while vein density would remain closely tied to genotype-specific differences in epidermal cell size. To test this we compared observed relationships in wild-type and mutant genotypes of *Arabidopsis* with modelled relationships that assume veins and stomata are passively 'diluted' by epidermal cell expansion.

5.3 Materials and methods

5.3.1 Plant material and growth conditions

All genotypes included in this study were in the Col-0 ecotype of *Arabidopsis thaliana* (L.) Heynh. and Col-0 was used as the control genotype. Seven previously described mutant and transgenic genotypes were used. Genotypes were chosen because of their overall wild-type appearance and the restriction of the manipulated genes to the stomata and epidermal cells. The four mutant genotypes were: *tmm-1* (Nadeau and Sack 2002), *basl-2* (Dong *et al.* 2009), *epf1-1* (Hara *et al.* 2007), and *epf1-1;epf2-1* (Hunt and Gray 2009), and the three transgenic genotypes were $SPCH_{pro}$::SPCH-YFP, $SPCH_{pro}$::SPCH 2-4A-YFP and SPCH SILENCE (Lampard *et al.* 2008; Dow *et al.* 2014a; Dow *et al.* 2014b). Seeds were surface-sterilized and stratified at 4 °C for 3 - 5 d in 0.15 % agarose solution and then sown directly into pots of size 3.25" × 3.25" × 3" filled with Pro-Mix HP soil (Premier Horticulture, Quakerstown, PA, USA) and supplemented with Scott's Osmocote Classic 14 - 14 - 14 fertilizer (Scotts-Sierra, Marysville, OH, USA). At 10 - 14 d, seedlings were thinned so that only one seedling per pot remained. Plants of all genotypes were grown to maturity in growth chambers where the conditions were as follows:

day:night cycle, 16 h:8 h; day:night temperature, 22:20 °C; light intensity, approximately 100 µmol m^{-2} s^{-1}. Additional Col-0 plants were grown under 50, 160 and 200 µmol m^{-2} s^{-1} of incident light.

5.3.2 Leaf size and anatomical traits

All leaves were scanned at 300 pixels per inch (dpi) using a Canon CanoScan CS8800F flatbed scanner (Sydney, Australia) and leaf size measured (mm^2) using ImageJ (Rasband 1997-2014). Vein density (mm mm^{-2}), stomatal density (mm^{-2}), stomatal size (mm^2), stomatal index, epidermal cell density (total epidermal cells per unit area; mm^{-2}), epidermal cell size (mm^2) and presence of stomatal clusters or pairs was then quantified from one rosette leaf from each of six plants per mutant or transgenic genotype (except *epf1;epf2* where four plant were sampled, and *epf1* and SPCH SILENCE where five plants were sampled) and one rosette leaf from each of eight, six, five and three Col-0 plants grown under 50, 100, 160 and 200 µmol m^{-2} s^{-1} of incident light, respectively. Leaves were cleared with a 7:1 ethanol:acetic acid solution overnight or longer, softened for 30 min in 1 M potassium hydroxide and rinsed with water. Leaves from the mutant and transgenic genotypes and Col-0 plants grown under 100 µmol m^{-2} s^{-1} of incident light were then stained with 1 % crystal violet and mounted on microscope slides in phenol glycerine jelly. Leaves from Col-0 plants grown under 50, 160 and 200 µmol m^{-2} s^{-1} of incident light were left unstained and mounted on microscope slides in Hoyer's solution.

Ten fields of view were photographed from all leaves to determine vein density. Care was taken to ensure representative sampling across the middle of the leaf and leaf margin (the midrib was avoided). Photomicrographs of leaves stained with crystal violet were taken at 4 × magnification (field of view area 3.47 mm^2) using a Nikon Digital Sight DS-L1 camera (Melville, NY, USA) mounted on a Leica DM 1000 microscope (Nussloch, Germany). Unstained leaves were visualised by differential interference contrast microscopy and photomicrographs taken at 5 × magnification

through a 0.7 × tube (field of view area 4.72 mm^2) using a Leica DFC450 digital microscope camera mounted on a Leica DM 2000 LED microscope. A further ten fields of view were photographed from both the abaxial and adaxial surfaces of all leaves to determine stomatal density, stomatal size, stomatal index, epidermal cell density, epidermal cell size and whether any stomata were present in clusters or pairs. As mentioned above sampling comprised a representative sample of the leaf surface except for the midrib. Leaves stained with crystal violet were photographed at 20 × magnification (field of view area 0.141 mm^2) and unstained leaves were visualised by differential interference contrast microscopy and photographed at 20 × magnification through a 0.7 × tube (field of view area 0.301 mm^2) using the same camera and microscope setups described above.

All leaf anatomical traits were quantified using ImageJ. Stomatal size was measured from five stomata (comprising a pair of guard cells) per field of view. Epidermal cell size (S_{EC}) was calculated as:

$$S_{EC} = (1 - (D_S \times S_S)) / D_{EC}, \quad (5.1)$$

where D_S is stomatal density, S_S is stomatal size and D_{EC} is epidermal cell density. Stomatal index (SI) was calculated as:

$$SI = (D_S / (D_S + D_{EC})) \times 100, \quad (5.2)$$

according to Salisbury (1928). Partial stomata and epidermal cells were included in density counts if visible along the top and right-hand border of photomicrographs and discarded if visible along the bottom and left-hand border. The presence of stomatal clusters or pairs was assessed visually from photomicrographs.

5.3.3 Statistical analysis

Mean abaxial and adaxial stomatal density, abaxial and adaxial epidermal cell size, abaxial and adaxial stomatal index, leaf size and ratio of vein density to 1/√abaxial epidermal cell size of the mutant and transgenic genotypes was compared with mean values of Col-0 plants grown under 100 µmol m^{-2} s^{-1} of incident light using unpaired *t*-tests. Mean abaxial and adaxial stomatal index of Col-0 plants grown under 50, 160 and 200 µmol m^{-2} s^{-1} of incident light were also compared with mean values from Col-0 plants grown under 100 µmol m^{-2} s^{-1} of incident light using unpaired *t*-tests. The correlation coefficient (r^2) and statistical significance of co-variation between parameters ($P < 0.05$) was then determined for the relationships between vein density and 1/√abaxial epidermal cell size, stomatal density and 1/epidermal cell size (on both leaf surfaces) and between vein density and √abaxial + √adaxial stomatal density across all Col-0 plants using R (R Core Team 2014). Epidermal cell size and stomatal density were transformed according to the expectation that cell expansion dictates vein and stomatal density. Analyses were performed at the plant level because there were highly significant pooled within treatment correlations between vein density and 1/√abaxial epidermal cell size, abaxial stomatal density and 1/abaxial epidermal cell size, adaxial stomatal density and 1/adaxial epidermal cell size and vein density and √abaxial + √adaxial stomatal density ($r^2 = 0.45$; $P < 0.01$, $r^2 = 0.81$; $P < 0.001$, $r^2 = 0.71$; $P < 0.001$ and $r^2 = 0.6$; $P < 0.001$, respectively with 17 degrees of freedom; i.e. $n - 5$ to allow for the loss of degrees of freedom from fitting means for four treatments). Note that the relationships within treatments were highly similar to those among treatments. The relative contribution of stomatal index and 1/epidermal cell size to the r^2 of the multiple regression in which they are predictors of stomatal density was also quantified for the abaxial and adaxial leaf surfaces in Col-0 plants as a relative importance metric (lmg) using the 'relimp' package in R.

5.3.4 'Passive dilution' models

The relationships between vein density, stomatal density and epidermal cell size in Col-0 plants were also compared with modelled relationships using ANOVA in R. Modelled relationships were calculated according to the method outlined in Chapter 3. The modelled relationships between vein density, stomatal density and epidermal cell size were based on the 'passive dilution' hypothesis whereby vein and stomatal density are coordinated by the expansion of epidermal cells. This assumes that vein and stomatal densities are uniquely related to epidermal cell size and that the epidermis comprises only epidermal and stomatal cells with a constant ratio between them (stomatal index). Thus, epidermal cell size was calculated for a range of stomatal densities using equation 5.1 where epidermal cell density was calculated as:

$$D_{EC} = (D_S / SI) - D_S, \quad (5.3)$$

using the mean stomatal index of the Col-0 plants grown under 100 µmol m^{-2} s^{-1} of incident light and the mean stomatal size across all plants (stomatal size was independent of changes to stomatal density (Fig. 5.1). This relationship was modelled separately for the abaxial and adaxial leaf surfaces.

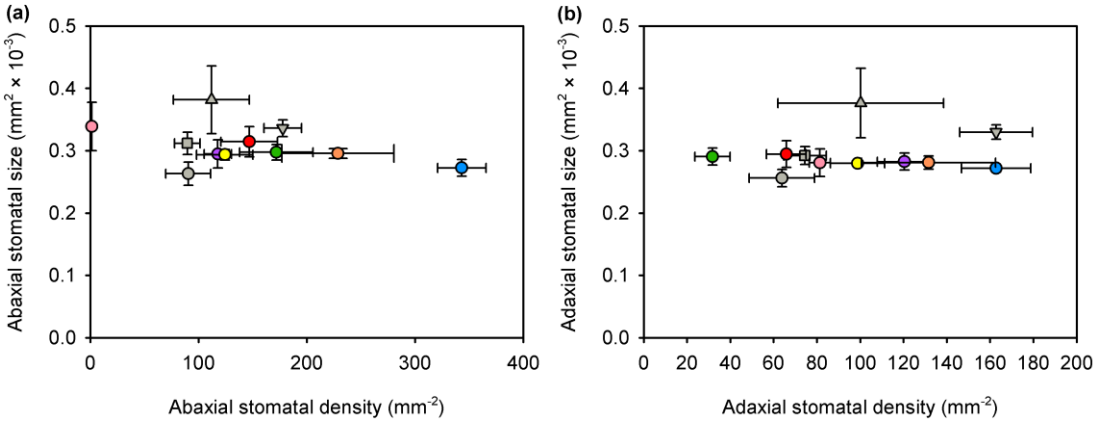

Figure 5.1 Mean stomatal density and size (± standard deviation) of Col-0 grown under four light treatments comprising 50, 100, 160 and 200 µmol m^{-2} s^{-1} of incident light (grey circles; *n* = 8, squares; *n* = 6, up-facing triangles; *n* = 5, and down-facing triangles; *n* = 3, respectively) and seven mutant and transgenic genotypes grown under standardised light conditions (100 µmol m^{-2} s^{-1} of incident light) (*basl*: purple circle, *epf1*: yellow circle, *epf1;epf2*: blue circle, SPCH 2-4A: orange circle, SPCH SILENCE: pink circle, SPCH-YFP: red circle and *tmm*: green circle) (*n* = 6 in all cases except *epf1* and SPCH SILENCE where *n* = 5 and *epf1;epf2* where *n* = 4).

The relationship between vein density and abaxial epidermal cell size was modelled using the simplified assumption that vein length is associated with a fixed proportion of the perimeter of an epidermal cell. According to this assumption one side of a theoretical square epidermal cell would always be in contact with vein tissue as it expanded. Thus, a geometric model of vein density as a function of abaxial epidermal cell size was determined for a fixed stomatal index (mean abaxial stomatal index of the Col-0 plants grown under 100 µmol m^{-2} s^{-1} of incident light) incorporating the mean abaxial stomatal size across all plants. Epidermal cell size was calculated as above. Assuming vein density (D_V) was a function of abaxial epidermal cell size we fitted the function:

$$D_V = a \times \text{abaxial } S_{EC}^{-0.5}, \quad (5.4)$$

where *a* is proportional to the epidermal cell perimeter associated with vein length. This returned a value of 0.1197 for *a*. This value was then used to predict the impact of abaxial epidermal cell size on vein density (using the equation vein density = 0.1197 × abaxial epidermal cell size$^{-0.5}$). Finally the expected relationship between vein density and √abaxial + √adaxial stomatal density was modelled by combining the relationships above.

5.4 Results

All mutant and transgenic genotypes had a different stomatal phenotype from Col-0 (Table 5.1). In some cases the effect of mutation or transgenes on phenotype was more severe on the abaxial leaf surface than on the adaxial surface. However, the leaves of all mutant and transgenic genotypes except SPCH SILENCE were the same size as Col-0 leaves.

Table 5.1 Phenotype of mutant and transgenic genotypes compared with Col-0 grown under 100 µmol m^{-2} s^{-1} of incident light

Genotype	Stomatal density (mm^{-2})		Epidermal cell size (μm^2)		Stomatal patterning		Leaf size (mm^2)
	abaxial	adaxial	abaxial	adaxial	abaxial	adaxial	
Col-0	**89.6 ± 4.8**	**74.5 ± 4**	**3490.9 ± 190.3**	**5198.6 ± 276.6**	**normal**	**normal**	**606.9 ± 44.4**
basl	117.7 ± 5.1 **	120.4 ± 5.1 ***	2791.6 ± 163.2 *	4503.5 ± 196.4 ns	clusters	clusters	522 ± 54.3 ns
epf1	124.3 ± 11.7 *	98.8 ± 5.6 **	3363.7 ± 196.5 ns	5404.7 ± 193.6 ns	normal	normal	578.7 ± 43.6 ns
epf1;epf2	343.1 ± 11.2 ***	162.8 ± 8 ***	1272.7 ± 102.7 ***	3888 ± 268.8 *	normal	normal	521.1 ± 45.9 ns
SPCH 2-4A	228.7 ± 21.1 ***	131.5 ± 12.7 **	895.1 ± 106.5 ***	3325.3 ± 329.5 **	normal	normal	533.4 ± 29.2 ns
SPCH SILENCE	1.4 ± 0.9 ***	81.4 ± 2.2 ns	4647.2 ± 186.9 **	3330.2 ± 186.8 ***	normal	normal	336.7 ± 40.1 **
SPCH-YFP	146.8 ± 11.7 ***	65.9 ± 4.1 ns	2033.2 ± 154.4 ***	5294.4 ± 266.2 ns	normal	normal	557.4 ± 27.2 ns
tmm	171.9 ± 13.8 ***	31.8 ± 3.3 ***	3900.9 ± 175.1 ns	6298.4 ± 179.7 **	clusters	pairs	633.8 ± 47.7 ns

Values are means ± standard error.

Symbols indicate a significant difference from Col-0 plants grown under 100 µmol m^{-2} s^{-1} of incident light (results of unpaired *t*-tests) (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, $P > 0.05$).

5.4.1 Co-variation with epidermal cell size contributes to the coordination of veins and stomata in Col-0

Light-induced changes to stomatal density and 1/epidermal cell size were coordinated on both leaf surfaces across the Col-0 plants (Fig. 5.2ab; abaxial: $r^2 = 0.79$; $P < 0.001$, adaxial: $r^2 = 0.75$; $P < 0.001$). However, the slope of the observed relationship was 78.7 % greater than the slope of the modelled relationship assuming stomatal density responds passively to epidermal cell expansion on the abaxial leaf surface and 67 % greater on the adaxial leaf surface ($P < 0.001$ in both cases). In

addition, stomatal index increased with light intensity on both leaf surfaces (Table 5.2). Despite this, 1/epidermal cell size was a more important determinant of stomatal density than stomatal index (explaining 57.2 % of variation in stomatal density on the abaxial leaf surface versus the 42.8 % explained by stomatal index and 56.5 % versus 43.5 % on the adaxial leaf surface). The relationship between vein density and 1/√abaxial epidermal cell size was also coordinated across Col-0 plants (Fig. 5.2c; $r^2 = 0.41$; $P < 0.01$), although the slope of the observed relationship was 35.8 % less than the slope of the modelled relationship assuming vein density is determined by epidermal cell expansion alone (i.e. there was not a constant ratio between vein density and 1/√abaxial epidermal cell size) ($P < 0.05$). Thus, changes to vein density and stomatal density (abaxial and adaxial leaf surfaces) were coordinated (Fig. 5.2d) ($r^2 = 0.75$; $P < 0.001$) but the slope of the observed relationship was 54.2 % less than modelled ($P < 0.001$).

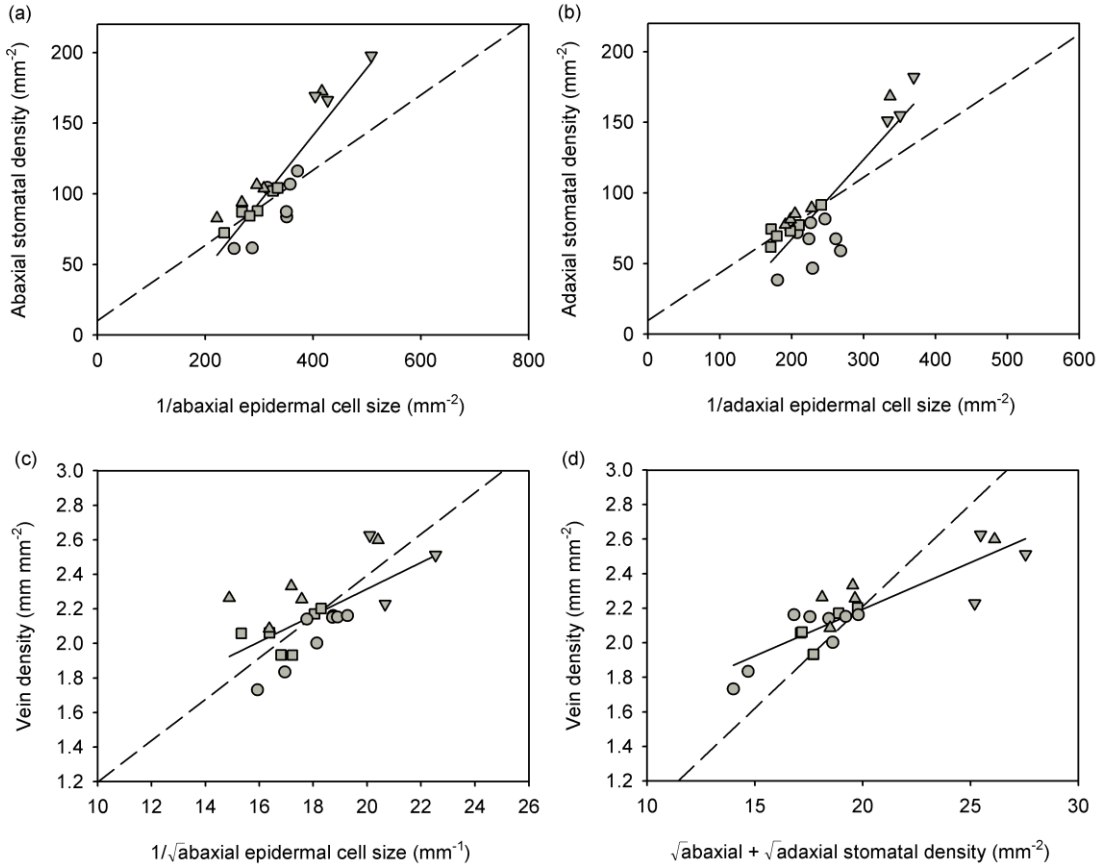

Figure 5.2 Observed relationships between stomatal density and 1/epidermal cell size (each symbol represents the mean vein and stomatal density per leaf from 10 counts) on the **(a)** abaxial and **(b)** adaxial leaf surfaces, **(c)** vein density and 1/√abaxial epidermal cell size and **(d)** vein density and √abaxial + √adaxial stomatal density across Col-0 plants grown under a range of light conditions (grey symbols and black solid lines) compared with modelled relationships (black dashed lines). Light treatments comprised 50, 100, 160 and 200 µmol m^{-2} s^{-1} of incident light (circles, squares, up-facing triangles and down-facing triangles, respectively). Modelled relationships assume veins and stomata are passively 'diluted' by epidermal cell expansion and that stomatal index is constant (see materials and methods for details). Black solid lines are regressions between stomatal density and 1/epidermal cell size (abaxial $D_S = 0.476 \times 1/\text{abaxial } S_{EC} - 48.948$, $r^2 = 0.79$, $P < 0.001$; adaxial $D_S = 0.564 \times 1/\text{adaxial } S_{EC} - 45.363$, $r^2 = 0.75$, $P < 0.001$), vein density and 1/√abaxial epidermal cell size ($D_V = 0.0769 \times 1/\sqrt{\text{abaxial}}\ S_{EC} + 0.779$, $r^2 = 0.41$, $P < 0.01$) and vein density and √abaxial + √adaxial stomatal density ($D_V = 0.0541 \times \sqrt{\text{abaxial}} + \sqrt{\text{adaxial}}\ D_S + 1.113$, $r^2 = 0.75$, $P < 0.001$). All observed relationships were significantly different from modelled relationships.

Table 5.2 Stomatal index of Col-0 plants grown under 50, 160 and 200 µmol m^{-2} s^{-1} of incident light compared with plants grown under 100 µmol m^{-2} s^{-1} of incident light

Light treatment (µmol m^{-2} s^{-1} of incident light)	**Stomatal index**	
	abaxial	**adaxial**
100	**23.4 ± 0.3**	**27.8 ± 0.8**
50	21.5 ± 1 ns	21.6 ± 1.4 **
160	27.3 ± 0.8 ***	30.6 ± 1 *
200	29.1 ± 0.4 ***	33.1 ± 0.6 **

Values are means ± standard error.

Symbols indicate a significant difference from Col-0 plants grown under 100 µmol m^{-2} s^{-1} of incident light (results of unpaired *t*-tests) (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, $P > 0.05$).

5.4.2 Vein density is independent of variation in epidermal cell size among mutant and transgenic genotypes

As predicted, relationships between stomatal density and 1/epidermal cell size on the abaxial and adaxial leaf surfaces differed from modelled relationships amongst the mutant and transgenic genotypes (Fig. 5.3ab). Genotypes that deviated the most from modelled relationships had higher or lower stomatal index than Col-0 plants grown under 100 µmol m^{-2} s^{-1} of incident light (Table 5.3), indicating that genetic controls determining stomatal index had a strong influence on stomatal density. However, contrary to the second part of our hypothesis, relationships between vein density and 1/√abaxial epidermal cell size dramatically deviated from the modelled relationship amongst the mutant and transgenic genotypes (Fig. 5.3c). Thus, the ratio of vein density to 1/√abaxial epidermal cell size in these genotypes differed from that observed in Col-0 plants grown under 100 µmol m^{-2} s^{-1} of incident light (Table 5.3),

demonstrating that vein density was not affected by large changes to epidermal cell size. Consequently, the relationship between vein density and stomatal density (abaxial and adaxial leaf surfaces) also deviated from the modelled relationship in some mutant and transgenic genotypes (Fig. 5.3d).

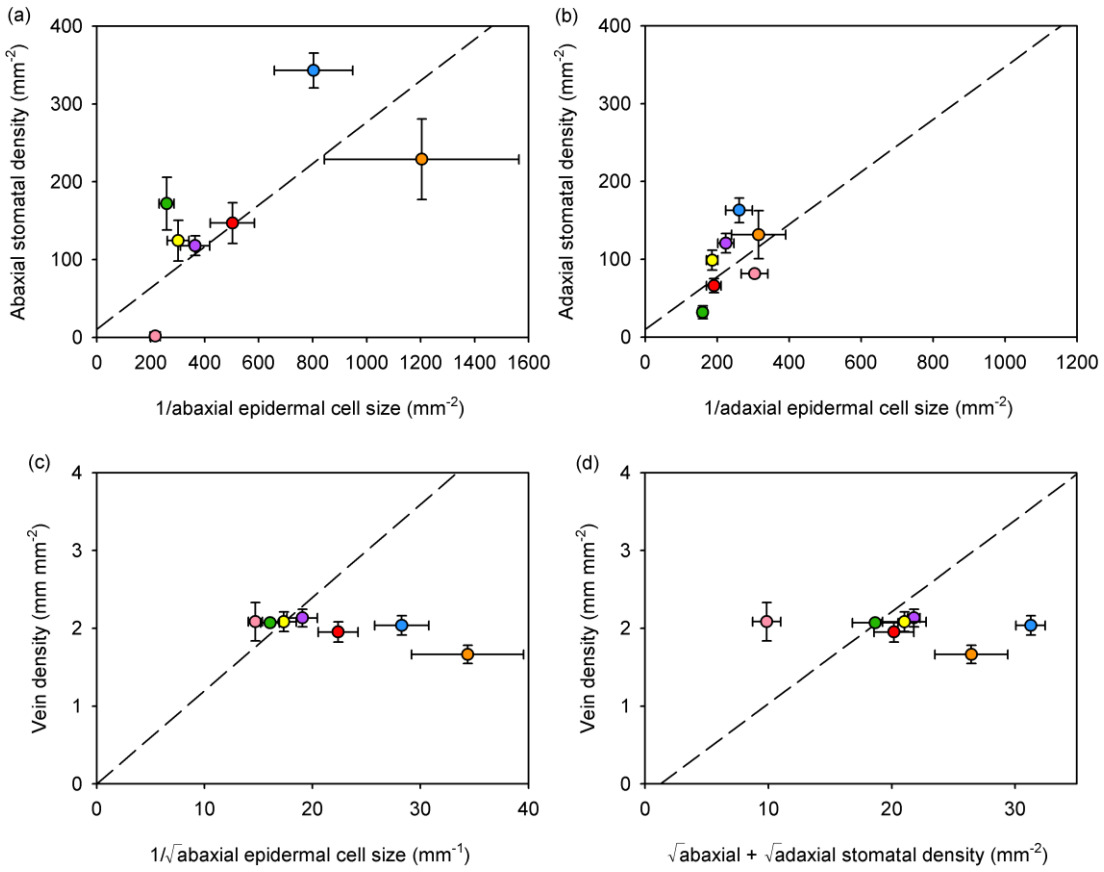

Figure 5.3 Stomatal density and 1/epidermal cell size on the **(a)** abaxial and **(b)** adaxial leaf surfaces, **(c)** vein density and 1/√abaxial epidermal cell size and **(d)** vein density and √abaxial + √adaxial stomatal density of seven mutant and transgenic genotypes grown under standardised light conditions (100 µmol m^{-2} s^{-1} of incident light) (*basl*: purple circle, *epf1*: yellow circle, *epf1;epf2*: blue circle, SPCH 2-4A: orange circle, SPCH SILENCE: pink circle, SPCH-YFP: red circle and *tmm*: green circle) compared with modelled relationships (black dashed lines). Symbols are means ± standard deviation (*n* = 6 in all cases except *epf1* and SPCH SILENCE where *n* = 5 and *epf1;epf2* where *n* = 4). Modelled relationships assume veins and stomata are passively 'diluted' by epidermal cell expansion and that stomatal index is constant (see materials and methods for details).

Table 5.3 Stomatal index and ratio of vein density to 1/√abaxial epidermal cell size of the mutant and transgenic genotypes compared with Col-0 plants grown under 100 µmol m^{-2} s^{-1} of incident light

Genotype	Stomatal index		Vein density:1/√ abaxial epidermal cell size
	abaxial	adaxial	
Col-0	**23.4 ± 0.3**	**27.8 ± 0.8**	**0.12 ± 0.0032**
basl	24.6 ± 0.6 ns	34.4 ± 0.5 ***	0.11 ± 0.0048 ns
epf1	29.4 ± 0.9 ***	34.5 ± 0.5 ***	0.12 ± 0.0027 ns
epf1;epf2	32 ± 1.1 ***	38.5 ± 0.8 ***	0.072 ± 0.0023 ***
SPCH 2-4A	16.6 ± 0.5 ***	29.9 ± 0.9 ns	0.048 ± 0.0027 ***
SPCH SILENCE	0.5 ± 0.3 ***	20.9 ± 0.9 ***	0.14 ± 0.0062 *
SPCH-YFP	22.8 ± 0.6 ns	25.6 ± 0.6 ns	0.089 ± 0.0013 ***
tmm	39.8 ± 1.1 ***	15.9 ± 1.1 ***	0.13 ± 0.0037 ns

Values are means ± standard error.

Symbols indicate a significant difference from Col-0 plants grown under 100 µmol m^{-2} s^{-1} of incident light (results of unpaired *t*-tests) (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, $P > 0.05$).

5.5 Discussion

5.5.1 Vein density is not causally linked to epidermal cell expansion

Epidermal cell size is a major determinant of vein and stomatal density in a diverse range of woody and herbaceous angiosperms and ferns (Chapters 3 and 4). Consequently, it has been proposed that developmental coordination of these traits is facilitated by a 'passive dilution' mechanism in which densities of veins and stomata

are co-regulated by epidermal cell size. However, the dramatic divergence of relationships between vein density and epidermal cell size in mutant and transgenic *Arabidopsis* genotypes from a modelled relationship that assumes vein density is passively 'diluted' by epidermal cell expansion (Fig. 5.3c) shows that variation in vein density is not causally linked to epidermal cell size. In contrast, epidermal cell size was a strong predictor of vein and stomatal density in Col-0 plants (despite some deviation from modelled relationships). This is supported by previous work that found stomatal and epidermal cell density both increase with increasing light intensity in *Arabidopsis* (Šantrůček *et al.* 2014). Thus, vein density was correlated with light-induced changes to epidermal cell size but was unresponsive to variation in epidermal cell size among genotypes.

5.5.2 Vein density may be regulated by cells that mirror epidermal cells

The independence of vein density from epidermal cell size in the mutant and transgenic genotypes and dependence on epidermal cell size in Col-0 plants grown under a range of light conditions suggests that vein density does not respond directly to differential epidermal cell expansion but rather to another factor that mirrors any light-induced changes to epidermal cell size. Cell sizes of independent leaf tissues are correlated across species suggesting that changes to cell size commonly occur in unison within the leaf (Brodribb *et al.* 2013; John *et al.* 2013). However, evidence from this study shows that abaxial epidermal cell size in mutant and transgenic genotypes of *Arabidopsis* can change independently from other leaf tissues. Because veins are embedded in the leaf mesophyll one would expect that mesophyll cell size may have a more direct influence on vein density. Thus, veins may be 'passively diluted' by mesophyll cell expansion and unified changes to mesophyll and epidermal cell size under sun and shade may drive the proportional relationships observed between vein density and epidermal cell size in wild type plants.

5.5.3 Leaf water potential may control cell size

We propose here that synchronised changes to the cell size of independent leaf tissues (the epidermis and mesophyll) coordinate the spacing of veins and stomata. As such we found that leaves from plants grown under low light had larger epidermal cells (and presumably larger mesophyll cells) than leaves from plants grown under higher light intensity. Cell expansion during leaf growth is sensitive to leaf water status, reviewed in Pantin *et al.* (2012). Thus, if shade leaves have higher apoplast water potential than sun leaves this would enable higher turgor pressure in growing tissue and induce greater cell expansion. In support of this view, it was found that shade leaves of herbs and woody angiosperms underwent greater epidermal cell expansion and had higher water potentials than sun leaves (Chapter 3). Furthermore, diurnal leaf expansion rates were depressed in *Arabidopsis* plants experiencing hydraulic limitation (Pantin *et al.* 2011).

5.5.4 Water supply and demand may be mismatched in some mutant and transgenic genotypes

There was considerable variation in stomatal density across the mutant and transgenic genotypes, but little variation in vein density. Presumably this resulted in a mismatch between water supply and demand in these genotypes as vein density is closely linked to leaf hydraulic conductance (Sack and Frole 2006; Brodribb *et al.* 2007) and stomatal density to stomatal conductance. In well-watered plants under normal VPD conditions an under supply of water through the veins would force stomata to close to prevent plant desiccation and an oversupply of water would result in overinvestment in veins and stomata. Thus, in both cases the carbon gain for energy investment in leaf infrastructure would be reduced. This would represent a significant disadvantage during competition with other more efficient genotypes. On the other hand, it may be advantageous to alter the ratio between vein and stomatal density under conditions that induce plant stress (e.g. drought or high VPD) (Chapter 2; Carins Murphy *et al.* 2014; Franks *et al.* 2015).

5.6 Conclusions

Contrary to our hypothesis, vein density was independent of variation in epidermal cell size among mutant and transgenic *Arabidopsis* genotypes. Conversely, epidermal cell size was an important predictor of vein density in wild type plants grown under a range of light conditions. Thus, we suggest that vein density is regulated by another cell type (most likely the mesophyll) that mirrors any light-induced changes to epidermal cell size. These results suggest that synchronised changes to cell size in different leaf tissues are required for the coordination of vein and stomatal density, and thus, for maintaining a balance between water supply and transpirational demand. One would expect that there is selective pressure to achieve this kind of coordination as a mismatched supply of water would result in significant costs to plants in terms of vein and stomatal infrastructure or reduced CO_2 uptake due to stomatal closure.

Chapter 6
General discussion

6.1 General discussion on key findings

The central finding of this thesis is that a constant ratio between leaf vein and stomatal density is maintained by their covariance with cell size (Fig. 6.1). This 'passive dilution' mechanism appears to co-regulate plastic changes to vein and stomatal density within species and adaptive changes across woody and herbaceous angiosperms and ferns. This suggests that adaptation favours coordination of veins and stomata by this mechanism over independent development of these tissues. This is further supported by the results of Chapter 5 which suggest that synchronised changes to the cell size of different leaf tissues (epidermis and mesophyll) are required to coordinate vein and stomatal density.

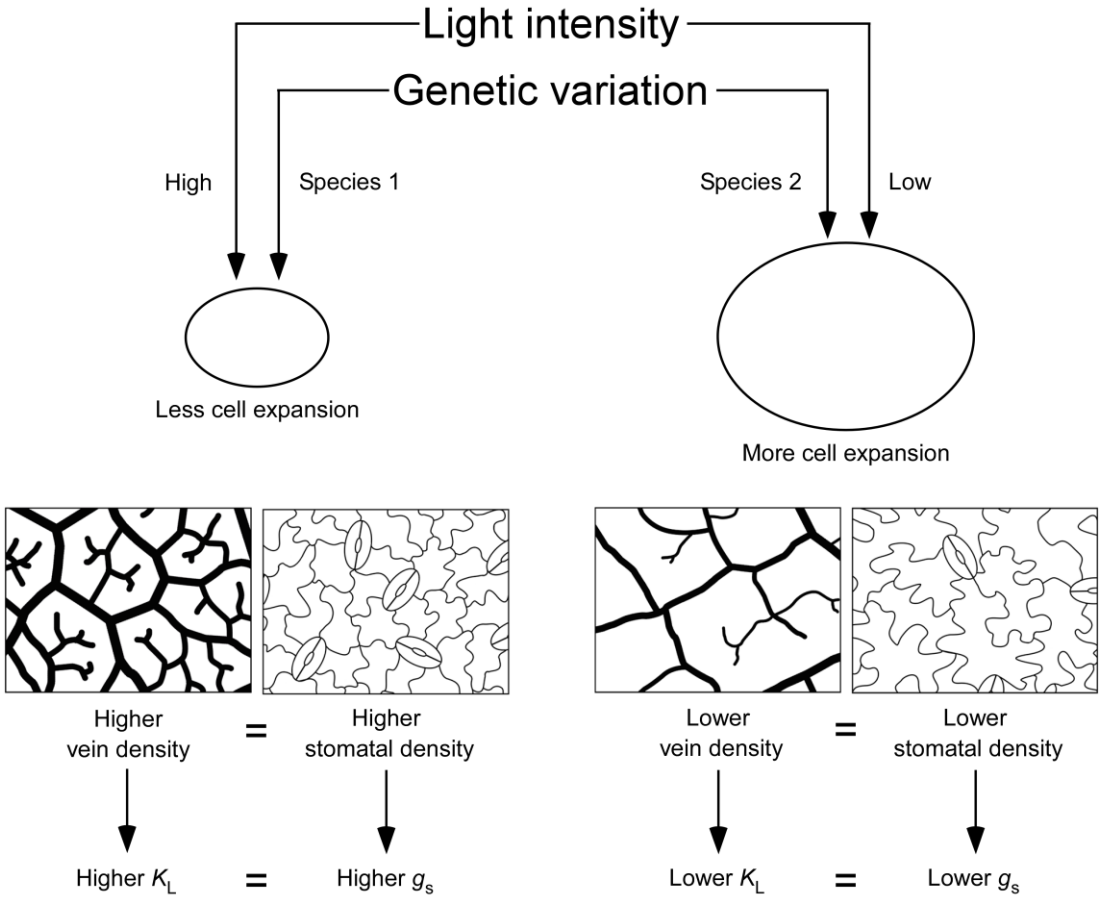

Figure 6.1 Differential cell expansion in response to variation in light intensity, or variation among species, 'dilutes' leaf veins and stomata in unison. This results in proportional co-variation of vein and stomatal density with cell size and a balance between water supply (leaf hydraulic conductance; K_L) and transpirational demand (stomatal conductance; g_s).

I found that differential epidermal cell expansion co-regulates plastic changes to vein and stomatal density within species as well as adaptive changes across species (Figs 3.3, 3.4 and 4.3). This means that greater epidermal cell expansion in the shade, and in some species compared with others, increases the space between veins and stomata reducing the density of both these structures in unison. In comparison, I found that the difference in vapour pressure between leaf tissue and the atmosphere (the VPD) has a minor effect on epidermal cell expansion (Table 2.1) and instead modifies how many cells are initiated (Fig. 2.4). Thus, the co-variance of vein and stomatal density with epidermal cell size - and perhaps mesophyll cell size (Chapter

5) - ensures that water supply and transpirational demand are balanced, which should maximise the benefit-cost ratio between photosynthetic output and plant investment in vein and stomatal infrastructure. These findings further clarify the results of previous research that found leaf expansion (mediated by epidermal cell size) co-regulated vein and stomatal density in woody angiosperms (Carins Murphy *et al.* 2012). They also provide a general mechanism that may underlie the developmental coordination observed between leaf vein and stomatal density within individual plants, within species, across populations of the same species and across species in a range of woody and herbaceous angiosperms and ferns (Brodribb and Jordan 2011; Zhang *et al.* 2012; Brodribb *et al.* 2013; Yang *et al.* 2014; Zhang *et al.* 2014; Fiorin *et al.* in press).

6.2 Applications and future directions

The proportional relationships between vein density, stomatal density and epidermal cell size can be used to make predictions about plant function and the conditions experienced during leaf development. This means leaves with small cells are likely to have high densities of veins and stomata, high leaf conductances to water in liquid- and vapour-phase, and high rates of transpiration and carbon dioxide (CO_2) assimilation (Brodribb *et al.* 2013). Because epidermal cell expansion is responsive to light intensity, this would be especially useful when comparing fossil leaf anatomy within individuals or species to differentiate sun and shade leaves. These relationships also have implications for predicting how plants may respond to changing environmental conditions. For example, differential cell expansion may facilitate leaf acclimation to other environmental factors in addition to light intensity. If so, greater flexibility in cell size may allow some species to acclimate to a wider range of conditions. However, exactly how the relationships between vein density, stomatal density and epidermal cell size respond to other important environmental factors that affect plant function such as atmospheric CO_2 concentration and temperature remains to be investigated.

Plants grown in above ambient atmospheric CO_2 concentration may exhibit a coordinated decrease in vein and stomatal density to maintain homeostasis in carbon assimilation and a balance between water supply and transpirational demand. A decrease in stomatal density would mitigate enhanced carbon uptake due to a steeper CO_2 concentration gradient between leaf tissue and the atmosphere, and vein density would be expected to mirror any changes in stomatal density to efficiently replace transpired water. This idea is supported by the observation that decreases in stomatal conductance and stomatal density in sunflower plants grown under elevated CO_2 concentration are associated with decreases in stem xylem specific conductivity and conduit size (Rico *et al.* 2013). However, it is evident from the literature that although growth under high CO_2 concentration generally induces a decrease in stomatal density it appears to have no effect on leaf vein density (Woodward 1987; Uhl and Mosbrugger 1999; Royer 2001; Boyce and Zwieniecki 2012). As the ratio of stomata to total epidermal cells (the stomatal index) also typically decreases in response to increasing CO_2 concentration (Woodward 1987; Royer 2001) then presumably vein density is limited by cell expansion which may be less responsive to CO_2 concentration than light intensity. If so, this would result in less efficient investment in leaf vasculature and effect plant performance under rising atmospheric CO_2 concentration. Consequently, plants with intrinsically high leaf vein densities may be disadvantaged.

Growth temperature is another environmental factor that may affect the relationship between vein and stomatal density. However, how temperature affects the ratio between these traits is not clear from the literature. Some studies report an increase in vein and stomatal density with increasing growth temperatures (Zhu *et al.* 2012; Hu *et al.* 2014), but several others observed decreases in vein and stomatal density with increasing temperature (Ciha and Brun 1975; Beerling and Chaloner 1993; Ghosh *et al.* 1996; Luomala *et al.* 2005; Muller *et al.* 2014; Li *et al.* 2015). This inconsistency may be a result of plants experiencing greater VPD at higher growth temperatures. In Chapter 2 I found that plants grown under high VPD had greater densities of veins

and stomata than plants grown under low VPD. Similar positive relationships between temperature and vein and stomatal density may be seen if VPD is not controlled. Thus, the negative relationship between temperature and vein and stomatal density observed in some studies may reflect variation in temperature rather than VPD. However, the influence of temperature on the development of veins and stomata needs to be observed under constant VPD to definitively separate the effects of these factors.

Understanding the effect of CO_2 concentration and temperature on the relationship between vein and stomatal density could inform the use of these parameters as proxies for past climates (Blonder and Enquist 2014). If the relationship between vein and stomatal density is maintained under variable light intensity and VPD, but modified by CO_2 concentration and/or temperature, then comparing how the ratio between vein and stomatal density has changed over time may provide some insight into past environmental conditions and patterns of plant evolution as well as how plants may respond to changing climatic conditions in the future.

6.3 Conclusions

In this thesis I demonstrate that vein and stomatal density co-vary with epidermal cell size both within and across species in a diverse range of woody and herbaceous angiosperms and ferns, and that this passive 'dilution' of veins and stomata by cell expansion facilitates their coordination. Furthermore, I show that vein density is not causally linked to epidermal cell expansion, and suggest that mesophyll cell expansion (in unison with epidermal cell expansion) may directly regulate vein density. These results provide insight into how plants balance water supply with transpirational demand to ensure maximum rates of carbon assimilation are achieved for the minimum investment in veins and stomata. This maximises the energetic return for investments made during leaf construction increasing the energy available for growth and reproduction.

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Appendix 1

Publication from PhD candidature

Reprint attachment of the paper published from this thesis corresponding to Chapter 2:

Carins Murphy MR, Jordan GJ, Brodribb TJ (2014) Acclimation to humidity modifies the link between leaf size and the density of veins and stomata. *Plant, Cell and Environment* **37**, 124-131.

Carins Murphy MR, Jordan GJ, and Brodribb TJ (2014) Acclimation to humidity modifies the link between leaf size and the density of veins and stomata. Plant cell and environment 37(1), 124-131.